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Comparative Composition of Bacteria in the Human Intestinal Microflora During Remission and Active Ulcerative Colitis

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Abstract

Ulcerative colitis is a severe, relapsing and remitting disease of the human large intestine characterised by inflammation of the mucosa and submucosa. The main site of disease is the sigmoid/rectal region of the large bowel but the aetiology remains unknown. There is considerable evidence to indicate that the components of the resident colonic microflora can play an important role in initiation of the disease. The present study was aimed at characterising the faecal microflora of ulcerative colitis patients in remission and active phases to determine profile differences. Faecal samples were obtained from 12 patients, 6 with active colitis and 6 in remission. The samples were analysed for populations of lactobacilli, bifidobacteria, clostridia, bacteroides, sulphate-reducing bacteria (SRB) and total bacteria using culture independent fluorescence *in situ* hybridisation (FISH). *Lactobacillus*-specific denaturing gradient gel electrophoresis (DGGE) was then performed to compare the species present. Numbers of lactobacilli were significantly lower ($p < 0.05$) during the active phase of the disease but the other populations tested did not differ. DGGE analysis revealed that *Lactobacillus salivarius*, *Lactobacillus manihotivorans* and *Pediococcus acidilactici* were present in remission, but not during active inflammation. These results imply that a reduction in intestinal *Lactobacillus* species may be important in the initiation of ulcerative colitis.

Introduction

Ulcerative colitis (UC) is a chronic inflammatory condition of the large intestine. Patients may present at any age, with men and women being equally affected. Current incidence in the West is around 10 cases per 100 000 population (Probert *et al.*, 1992). It is characterised by acute non-infectious inflammation of the colonic mucosa, manifesting physically as rectal bleeding and/or bloody diarrhoea and often accompanied by left-sided abdominal pain (Ghosh *et al.*, 2000). The aetiology of the disease is largely unknown. However, several studies have shown that the presence of a luminal microflora is a necessary

co-factor for the disease in animal models of colitis (Hans *et al.*, 2000; Rath *et al.*, 1996).

Specific species of bacteria have previously been implicated in the pathogenesis of UC. Induction of gastrointestinal inflammation has been observed in rodent models of colitis mono-associated with *Bacteroides vulgatus* (Rath *et al.*, 2001; Setoyama *et al.*, 2003). *B. vulgatus* was also found in greater numbers in colonic biopsies taken from UC patients when compared to healthy individuals (Matsuda *et al.*, 2000). Serum antibody responses against *B. vulgatus* and *B. fragilis* were also higher in these patients. There is an indication that *Bacteroides* may express a specific outer membrane protein that triggers an inflammatory response (Bamba *et al.*, 1995).

Mycobacterium avium subsp. *paratuberculosis* has been associated with granulomatous Crohn's Disease (Sanderson *et al.*, 1992; Suenaga *et al.*, 1999), although there is no evidence to firmly link this organism with UC (Sutton *et al.*, 2000). *Clostridium difficile* toxin has been found to exacerbate inflammation in patients with chronic colitis (Sartor *et al.*, 1996).

Research has suggested that sulphate-reducing bacteria (SRB) are involved in UC as their metabolic end product, hydrogen sulphide, is a highly cytotoxic compound (Pitcher and Cummings, 1996; Roediger *et al.*, 1997). This may act through inhibition of butyrate oxidation, the primary energy source for colonocytes, leading to cell death and chronic inflammation. Studies have shown that the prevalence of SRB in the faeces of patients with UC (100% of patients tested) is significantly higher than healthy individuals (approximately 50%) (Gibson *et al.*, 1988; Pitcher *et al.*, 1995). Specific species such as *Desulfovibrio piger* have been detected in the colon of a high number of UC patients (Loubinoux *et al.*, 2002). Zinkevich and Beech (2000) also demonstrated the ubiquitous presence of SRB in the colitic human colon mucosa.

Amelioration of the symptoms of UC in both experimental models and human patients has been produced by administration of probiotic bacterial species. Germ-free interleukin (IL)-10 gene-deficient mice pre-treated with *L. plantarum* developed significantly less severe colitis when subsequently exposed to a specific pathogen free flora (Schultz *et al.*, 2002). A reduction in mucosal inflammatory activity was also observed with *L. salivarius* subspecies *salivarius* in the same model system (O'Mahony *et al.*, 2001). Bifidobacteria-fermented milk was shown to maintain remission when given as a dietary adjunct to UC patients, possibly reducing the relative proportion of *B. vulgatus* through local alterations in organic acid concentration (Ishikawa

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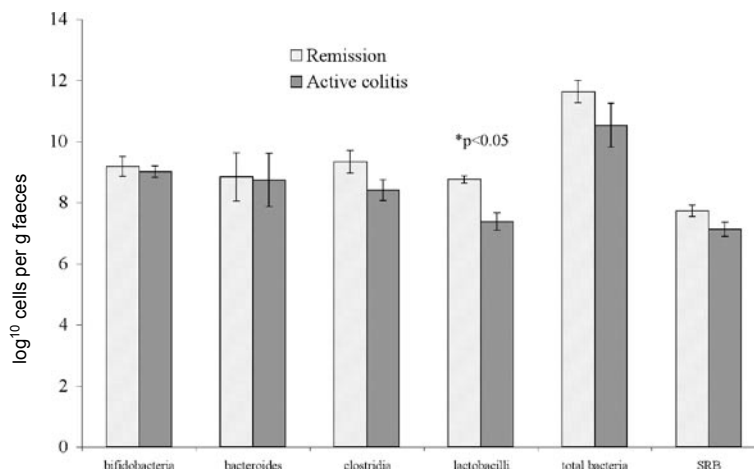


Figure 1. Counts of bacteria found in the faeces of ulcerative colitis patients (remission versus active disease).

et al., 2003). A mixed probiotic preparation containing *L. casei*, *L. plantarum*, *L. acidophilus*, *L. delbruekii* subspecies *bulgaricus*, *B. longum*, *B. breve*, *B. infantis* and *Streptococcus salivarius* subspecies *thermophilus* (VSL#3) was effective in maintaining remission in UC patients intolerant to conventional 5-aminosalicylic acid treatment (Venturi *et al.*, 1999). The resulting increase in colonic concentrations of lactobacilli and bifidobacteria was subsequently found to normalise colonic physiological function and barrier integrity (Madsen *et al.*, 2001).

The study reported here was designed to explore the colonic microflora of UC patients and discern whether there were any appreciable differences between bacterial populations in remission and active inflammation. Bacterial groups of interest were further investigated with molecular characterisation (DGGE), to discern microbial variation and thereby identify possible targets for probiotic or prebiotic intervention.

Results

FISH analysis of faecal samples revealed a significant ($p < 0.05$) decrease in total *Lactobacillus* numbers from patients in remission when compared to those with active colitis (Figure 1). Total bacteria were also reduced during inflammation, although the difference was not significant. There were no discernible differences in populations of the other bacteria tested.

As lactobacilli were the only group demonstrating appreciable differences between the two study groups, these were further explored using DGGE. Following PCR with *Lactobacillus*-specific primers (Figure 2), DGGE and sequence analysis revealed that *Lactobacillus salivarius*, *Lactobacillus manihotivorans* and *Pediococcus acidilactici* were present in remission, but not during active inflammation (Figure 3). Other species of bacteria found in the faecal samples of both patient groups were *L. casei*, *L. acidophilus*, *L. plantarum*, *L. ruminis*, *L. mucosae*, *L. delbruekii* subsp. *lactis*, *L. crispatus*, *L. gasseri*, and *L. paracasei*.

Discussion

Patients with UC tend to modify their diet and avoid certain foods that might trigger an inflammatory response (e.g. wheat products, alcohol, high fibre or complex carbohydrates) (Cashman and Shanahan, 2003). It is also documented that modifications in the dietary intake of certain of these components can affect colonic bacterial populations (Cresci *et al.*, 1999; Ballongue *et al.*, 1997; McBurney *et al.*, 1988). Thus, it was considered appropriate to compare the microflora profiles of patients with UC with those in remission, as they would likely be following a similar diet.

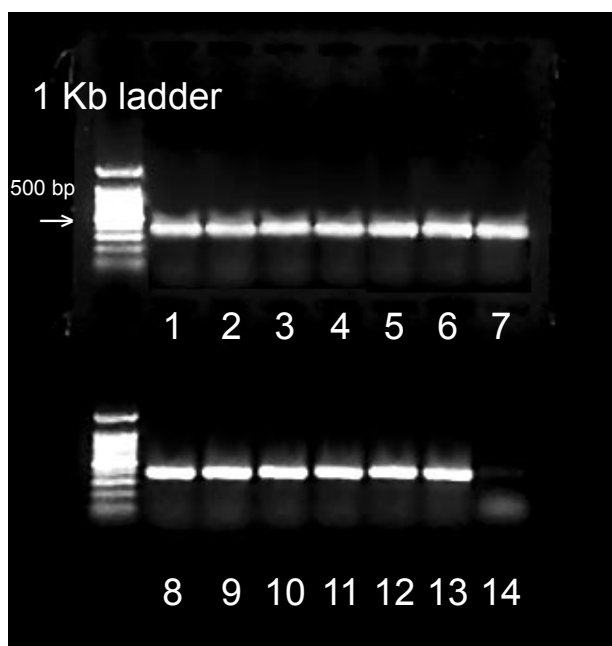


Figure 2. PCR from patients faecal samples with *Lactobacillus*-specific DGGE primers (340 bp fragment).

Key:
 samples 1→6 = remission;
 samples 7→12 = active colitis;
 sample 13 = +ve control *Lactobacillus acidophilus*
 sample 14 = -ve control MilliQ water

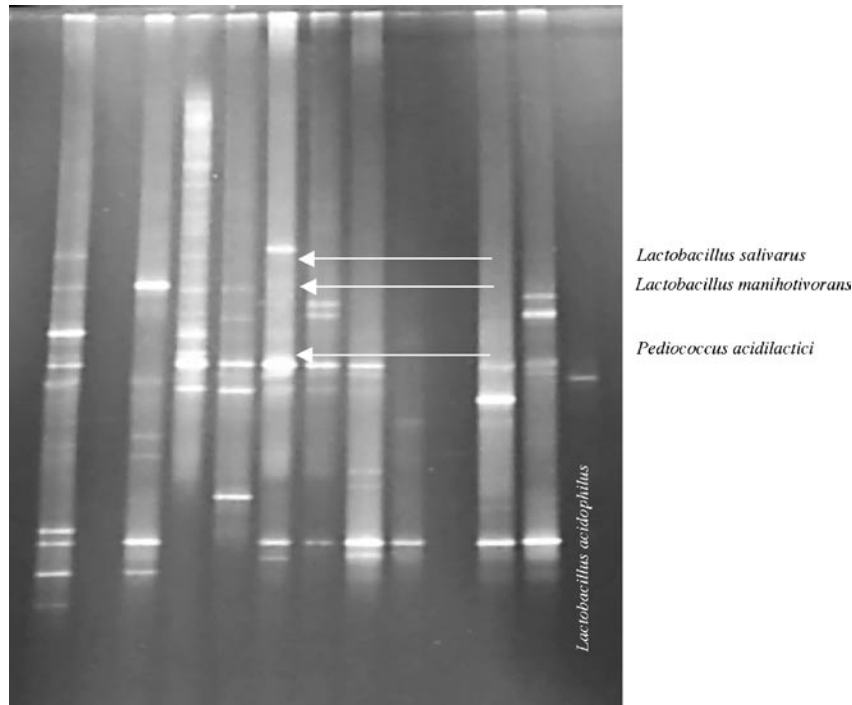


Figure 3. *Lactobacillus*-specific DGGE analysis of faecal samples from UC patients in remission and with active colitis. Key: samples 1→6 = remission; samples 7→12 = active colitis.

There was no significant difference between numbers of SRB in the two study groups. However, sulphate-reducing species were slightly more numerous in patients with active colitis when expressed as a percentage of the total bacterial population (data not shown). This is in agreement with previous studies of faecal samples taken from patients with active and quiescent UC (Pitcher *et al.*, 2000).

Numbers of lactobacilli were significantly reduced in patients with active colitis. Previous work has shown a decrease in lactobacilli concentrations in colonic biopsies from patients with active UC (Fabia *et al.*, 1993a; Pathmakanthan *et al.*, 1999). *Lactobacillus salivarius* subspecies *salivarius* has been shown to attenuate symptoms of colitis when fed to a murine model by reducing systemic and mucosal cytokine production (McCarthy *et al.*, 2003). Neonatal IL-10 deficient mice were found to have decreased levels of colonic lactobacilli and increased mucosal adherent and translocated bacteria. Normalising lactobacilli levels by daily rectal delivery of *L. reuteri* prevented the development of colitis (Madsen *et al.*, 1999). Similar results were obtained with intracolonic administration of *L. reuteri* in rats (Fabia *et al.*, 1993b).

Mechanistically, studies suggest that probiotic bacteria may reduce colonic inflammation by enhancing natural and acquired immunity. *L. rhamnosus*, *L. acidophilus* and *B. lactis* fed to mice increased the phagocytic activity of peripheral blood leucocytes and peritoneal macrophages, and significantly elevated splenic interferon- γ production (Gill *et al.*, 2000). Orally administered *L. casei* and *L. bulgaricus* were able to activate macrophages in mice,

inferring that probiotic bacteria could enhance the host immune response (Perdigon *et al.*, 1986). Different lactic acid bacteria were shown to produce distinct mucosal cytokine profiles in BALB/c mice. Increased IL-10 and IL-4 was observed mainly in mice fed with *L. delbrueckii* subspecies *bulgaricus* and *L. casei*, while a significant induction of IL-2 and IL-12 was observed with *L. acidophilus* (Perdigon *et al.*, 2002). If lactic acid bacteria are able to suppress populations of pathogenic species, such as *Bacteroides* or SRB, through stimulation of the host immune response, it might be expected that a reduction in colonic lactobacilli may elicit an inflammatory reaction and perhaps trigger the onset of active colitis.

In summary, the present data point to *Lactobacillus* species as a promising prebiotic target or probiotic therapy for the treatment of ulcerative colitis. Interventions in diet-matched patient groups are necessary to substantiate the evidence thus found. At present, DGGE primers are being designed to thoroughly explore changes in pathogenic bacteroides, clostridia and SRB populations in this and other studies of UC.

Experimental Procedures

Faecal samples

Studies were approved by the ethics committee of the Royal Berkshire Hospital NHS Trust. All subjects were attending outpatient clinics at the Department of Gastroenterology, Royal Berkshire Hospital, Reading, Berkshire. Faecal samples were obtained fresh from 12 ulcerative colitis patients, aged 32 to 73 years. 6 were in active and 6 in quiescent disease states. Of those with

Table 1. Oligonucleotide probes used in FISH analysis of faecal bacteria.

Target	Sequence 5'→3'	Position
bifidobacteria	CATCCGGCATTACCACCC	164
lactobacilli	GGTATTAGCA(C/T)CTGTTTCCA	158
bacteroides	CCAATGTGGGGACCTT	303
clostridia	TTTCC(C/T)TCTAATTATGGCGTATT	150
SRB	TACGGATTTCACTCCT	687

active colitis (age range 38 to 47 years, 2 female and 4 male), 3 were taking Prednisolone alone, 1 was taking Prednisolone plus oral Mesalazine and 2 were taking Mesalazine plus Azothioprine. All patients in remission (age range 32 to 73 years, 3 female and 3 male), were being prescribed oral Mesalazine and 1 patient was also taking Prednisolone. All patients admitted to avoiding a high vegetable fibre intake, with little or no alcohol consumption.

FISH

A 10% w/v dilution of each faecal sample was made with sterile, anaerobic phosphate buffered saline (PBS, pH 7.0) and aliquots fixed for 4h at 4°C in 4% w/v paraformaldehyde. Samples were centrifuged at 20,000 x g for 5 min and the pellet washed twice in sterile PBS. The final clean pellet was then re-suspended in 150 µl sterile PBS and 150 µl 100% ethanol. Samples were stored for a minimum of 1 h at -20°C prior to hybridisation. Genotypic 5'CY3-labelled 16S rRNA probes targeting bifidobacteria, bacteroides, clostridia (*Clostridium perfringens/histolyticum* subgroup), lactobacilli/enterococci and SRB were used (Table 1). The nucleic acid stain 4',6-diamino-2-phenylindole (DAPI) was employed for total bacterial counts. For each probe, 8 µl of cell suspension was diluted in 132 µl pre-warmed sterile hybridisation buffer (30 mM Tris-HCl, 1.36 M NaCl, 0.15% SDS, pH 7.2). 135 µl of this mixture was then added to 15 µl appropriate probe (50 nM). Hybridisations were carried out at 50°C (bifidobacteria and clostridia) and 45°C (lactobacilli and bacteroides) for 16 h. Sulphate-reducing bacteria were labelled for 4 h at 48°C. Following incubation, an appropriate dilution of each cell preparation was made in 5 ml sterile washing buffer (20 mM Tris-HCl, 0.9 M NaCl, pH 7.2). Separate hybridisation (20 mM TRIS-HCl, 0.9 M NaCl, 0.01% w/v SDS, 10% v/v formamide, pH 7.2) and washing (20 mM TRIS-HCl, 0.386 M NaCl, 0.01% w/v SDS, pH 7.2) buffers were used for the SRB probe. The suspensions were then filtered onto 0.2 µm black filter discs (Millipore) and mounted onto glass slides. Cells were enumerated by epifluorescent microscopy (510-560 nm). Comparison of the target bacterial groups was carried out using the Student t-test, with a significance level of p<0.05.

DGGE

Chromosomal DNA was extracted from the diluted faecal samples using a commercial kit (QiaAMP DNA Stool Mini Kit, Qiagen). Amplification of a 340-bp fragment was carried out using a PTC-200 DNA Engine (MJ Research) and the genus-specific primers Lac1 and Lac2GC (Walter *et al.*, 2001). The reaction mixture

(50 µl) contained 25 pmol of each primer, 0.2 mM of each deoxyribonucleotide and reaction buffer with 15 mM MgCl₂ (Roche Diagnostics). Approximately 100 ng DNA was used (typically between 1 and 2 µl) per reaction. 2.5 U Taq polymerase was added after an initial denaturation of 94°C for 2 min. PCR amplification was performed as follows: 35 cycles of 94°C for 30 s, 61°C for 1 min, 68°C for 1 min and 68°C for 7 min. Aliquots of the amplification product (5 µl) were analysed by gel electrophoresis in 1% w/v agarose containing ethidium bromide (0.5 µg/ml).

DGGE was performed using an Ingenuity PhorU-2 system. 10 µL of PCR samples were applied directly onto an 8% w/v polyacrylamide gel in 0.5 x TAE (20 mM Tris-acetate (pH 7.4), 10 mM acetate and 0.5 mM Na₂EDTA) stock solutions was formed with 8% w/v acrylamide stock solutions (acrylamide /N,N'-methylene bisacrylamide, 37:1, w/w) containing 20% and 80% denaturants (100% denaturant being 7 M urea and 40% v/v formamide). Electrophoresis was performed at a constant voltage of 100 V and a temperature of 60°C for 16 h. Following electrophoresis, the gel was stained for 1 h in Milli-Q water containing 0.5 µg/ml ethidium bromide and destained in Milli-Q water for 75 min. The gel was then visualised and photographed under UV (302 nm) transillumination (Kodak Digital Science v1D). Bands of interest were excised and the gel pieces soaked in 20 µl Milli-Q water for 4 h at 4°C. PCR was performed with Lac1 and Lac2GC primers as previously described, using 2 µl of the gel extract solution as DNA template. Amplicons were verified by gel electrophoresis. A 10 µl sequence mix was then prepared using 2 µl of the resultant PCR product, 1 µl of a 3.2 pmol/µl Lac1 primer solution, 3 µl MilliQ water and 4 µl BigDye v3.1 terminator mix (Applied Biosystems, UK). The sequencing reaction was performed as follows: 25 cycles of 96°C for 10 s, 50°C for 5 s and 60°C for 4 min. Products were sequenced via an ABI Prism 3700 capillary sequencer (Applied Biosystems, UK). Sequences retrieved were of a minimum length of 320-bp (data not shown). Strains were identified by comparison with known sequences obtained from GenBank and EBI databases via a BLAST search (Maidak *et al.*, 1997) at >99.5% sequence homology.

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