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ORIGINAL ARTICLE

Fluctuations in butyrate-producing bacteria in ulcerative colitis patients of North India

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Abstract

AIM: To study the interplay between butyrate concentration and butyrate-producing bacteria in fecal samples of ulcerative colitis (UC) patients *vs* control individuals.

METHODS: Fecal samples were collected from 14 control individuals (hemorrhoid patients only) and 26 UC patients (severe: n = 12, moderate: n = 6, remission: n = 8), recruited by the gastroenterologist at the Department of Gastroenterology, All India Institute of Medical Sciences, New Delhi, India. Disease activity in UC patients was determined by clinical colitis activity index. We employed fluorescent *in situ* hybridization in combination with flow cytometry to enumerate the clostridium cluster population targeted by *16S rRNA* gene probe. Major butyrate-producing species within this cluster were quantified to see if any change existed in control *vs* UC patients with different disease activity. This observed change was further validated by quantitative polymerase chain reaction. In addition to this, we carried out gas chromatography to evaluate the changes in concentration of major short chain fatty acids (SCFAs), namely acetate, *n*-butyrate, *iso*-butyrate, in the above samples. Student t test and Graph pad prism-6 were used to compare the data statistically.

RESULTS: There was a significant decrease of *Clostridium coccoides* (control, 25.69% ± 1.62% *vs* severe, 9.8% ± 2.4%, P = 0.0001) and *Clostridium leptum* clusters (control, 13.74% ± 1.05% *vs* severe, 6.2% ± 1.8%, P = 0.0001) in fecal samples of UC patients. Furthermore, we demonstrated that some butyrate-producing members of the clostridial cluster, like *Fecalibacterium prausnitzii* (control, 11.66% ± 1.55% *vs* severe, 6.01% ± 1.6%, P = 0.0001) and *Roseburia intestinalis* (control, 14.48% ± 1.52% *vs* severe, 9% ± 1.83%, P = 0.02) were differentially present in patients with different disease activity. In addition, we also demonstrated decreased concentrations of fecal SCFAs, especially of *n*-butyrate (control, 24.32 ± 1.86 mmol/μL *vs* severe, 12.74 ± 2.75 mmol/μL, P = 0.003), *iso*-butyrate (control, 1.70 ± 0.41 mmol/μL *vs* severe, 0.68 ± 0.24 mmol/μL, P = 0.0441) and acetate (control, 39.51 ± 1.76 mmol/μL *vs* severe, 32.12 ± 2.95 mmol/μL, P = 0.047), in the fecal samples of UC patients. The observed decrease of predominant butyrate producers of clostridial clusters correlated with the reduced SCFA levels in active UC patients. This was further confirmed by the restoration in the population of some butyrate producers with simultaneous increase in the level of SCFA in remission samples.

CONCLUSION: Our observations indicate that decreases in members of the clostridial cluster resulting in reduced butyrate levels contribute to the etiology of UC.

Key words: Fecal microbiota; Ulcerative colitis; Short chain fatty acids; Clostridial cluster; Fluorescent *in situ* hybridization-flow cytometry; Quantitative polymerase chain reaction

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INTRODUCTION

Inflammatory bowel disease (IBD), comprising of Crohn's disease and ulcerative colitis (UC), is a class of chronic inflammatory disorders of the intestine. An increasing trend in the incidence and prevalence of IBD in the Asian population has been recognized for the past two decades[1]. The dynamic balance between commensal microbiota and host defensive responses at the mucosal frontier has a pivotal role in the initiation and pathogenesis of chronic IBD[2]. Whether the exaggerated immune response is exerted to all commensal bacteria, or to a subset or a single strain of bacteria, is not known[3]. Differences in fecal microbiota of healthy subjects and IBD patients have been enumerated using different techniques[4-6]. Impaired cellular metabolism, such as butyrate oxidation and short chain fatty acid (SCFA) fermentation, has shown strong association with altered gut microbiota in UC patients[7,8].

SCFAs, such as acetate, propionate and butyrate, are produced by intestinal microbial fermentation of mainly undigested dietary carbohydrates, specifically resistant starches and dietary fiber, but also in a minor part generated by dietary and endogenous proteins in the intestine[9]. SCFAs are important for normal intestinal biology[10]. They also stimulate colonic sodium and fluid absorption and exert proliferative effects on the colonocytes[11]. Therefore, monitoring the fluctuations in SCFA concentration may help in understanding the relation of dysbiosis with UC.

Members of *Clostridium leptum* (*C. leptum*) and *Clostridium coccoides* (*C. coccoides*) groups together constitute the majority of Firmicutes (low G + C content bacteria)[12], producing large amounts of butyrate that function as an energy source for colonic epithelial cells and inhibit mRNA expression of proinflammatory cytokines in the mucosa by inhibiting nuclear factor-κB (NF-κB) activation[13]. Butyrate has been reported to help in prevention of colorectal cancer[14]. Evidence indicates that bacteria related to *Eubacterium hallii* (*E. hallii*), *Roseburia* species and *Eubacterium rectale* (*E. rectale*) within cluster XIVa and *Faecalibacterium prausnitzii* (*F. prausnitzii*)-related bacteria within cluster IV are normally the two most abundant groups of human fecal bacteria that produce butyrate[15]. These species-level probes account for a mean of 7.7% of the population of the total human fecal microbiota[16]. However, both the clostridial clusters harbor a diverse collection of several species of butyrate producers and non-butyrate producers as well[17].

Dynamics of predominant butyrate producers at the species level during disease activity and their correlation with the fluctuation in SCFA have not been established clearly. Here we explored the alteration in population of dominant butyrate-producing bacterial species present in fecal samples of UC patients at different disease stages. Abundance of the butyrate-producing clostridial cluster group was estimated by fluorescent *in situ* hybridization (FISH) in combination with flow cytometry and real time polymerase chain reaction (PCR). Further enumeration was carried out for known predominant members of butyrate-producing bacteria such as (1) *F. prausnitzii* as a member of the *C. leptum* group; and (2) *E. hallii* and *Roseburia intestinalis* (*R. intestinalis*) as members of the *C. coccoides* group. We also checked the level of butyrate, *iso*-butyrate and acetate in fecal samples to see if any correlation exists with butyrate-producing bacteria during different disease conditions.

MATERIALS AND METHODS

Fecal sample collection and processing

Disease activity in UC patients was determined by simple clinical colitis activity index[18] and the patients with total index score of 7-10, > 10 and 0-2 were assigned moderate, severe and remission category, respectively[19]. The patients were recruited by the gastroenterologist at the Department of Gastroenterology of the All India Institute of Medical Sciences, New Delhi, India. Clinical and demographic features of UC patients and controls are shown in Table 1. Patients who had hemorrhoids only and showed no evidence of small and large intestinal disease were enrolled as control individuals in this study. No study patient had received any antibiotic treatment in the past three months before sample collection. Patients under any antibiotic or probiotic treatments were excluded from this study. The fecal samples were collected in sterile stool specimen containers and stored at -80 ℃ within 3 h of sample collection until further processing.

Probes and oligonucleotides

All probes were designed from the *16S rRNA* gene. EUB 338 conserved within the bacterial domain was used as a positive control probe[20]; conversely NONEUB338 (reverse of EUB)[21] was used as a negative control probe. The positive control probe was double labeled with fluorescein isothiocyanate (FITC) at both the 5' and 3' end[22], whereas the negative control probe was labeled with FITC at the 5' end and Cy5 at the 3' end. We used two group-specific probes labeled with Cy5 at their 5' end (Sigma, India) and three species-specific probes labeled with FITC at their 5' end (Sigma, India). Two competitors (unlabeled) and 5 helper oligonucleotides (unlabeled) were used to increase the accessibility of the Clep 1156 probe[23] (Table 2).

Analysis of fecal samples by FISH-flow cytometry

About 1 g of fecal sample was suspended in 9 mL of phosphate buffered saline (PBS) and vortexed with 10-15 glass beads for 5 min to homogenize the sample. This suspension was centrifuged at 1500 rpm for 1 min

Table 1 Clinical and demographic features of ulcerative colitis patients and controls *n* (%)

Feature	UC (*n* = 26)	Control (*n* = 14)
Sex (F/M)	11 (42.30)/15 (57.69)	7 (50)/7(50)
Age at diagnosis (yr)		
mean ± SD	38.35 ± 11.49	35.00 ± 14.14
15-40	18 (69.23)	8 (57.14)
> 40	8 (30.76)	6 (42.85)
Disease behavior		
Severe	12 (46.15)	-
Moderate	6 (23.07)	-
Remission	8 (30.76)	-
Disease extent		
Proctitis	4 (15.38)	-
Left sided colitis	6 (23.00)	-
Pancolitis	6 (23.00)	-
None of the above	10 (38.46)	-
Smoking history		
Yes	3 (11.53)	2 (14.28)
No	23 (88.46)	12 (85.70)
Treatment history		
Immunosuppressant	16 (61.53)	-
Steroids	14 (53.84)	-
Appendectomy Y/N	4 (15.38)/22 (84.61)	0/14
Family history Y/N	2 (7.69)/24 (92.30)	0/14

UC: Ulcerative colitis; F: Female; M: Male; Y: Yes; N: No.

to pellet down the debris and the supernatant was collected. To fix the cells, 1 mL of this supernatant was incubated with 4% paraformaldehyde (1:3 ratio) at 4 ℃, overnight. The fixed cells were washed twice with PBS and incubated in ethanol-PBS solution (1:1 ratio) at -20 ℃ for 2 h. For each hybridization reaction, 60 μL of fixed cells were used. The fixed cells were washed twice with PBS and resuspended in 50 μL hybridization buffer (900 mmol/L NaCl, 20 mmol/L Tris-HCl of pH 8, and 0.01% sodium dodecyl sulfate at pH 7.2). All hybridizations were performed in the dark at 50 ℃ for 16 h in the hybridization solution containing 4 ng/μL of the appropriate labeled probe. One hundred and fifty microliter of hybridization solution (without probe) was added to stop the reaction and cells were pelleted at 1610 *g* for 10 min. Hybridized cells were further resuspended in prewarmed washing buffer [65 mmol/L NaCl, 20 mmol/L Tris-HCl, 5 mmol/L diaminoethanetetraacetic acid (EDTA), 0.01% sodium dodecyl sulfate, pH 7.2] and incubated at 50 ℃ for 20 min to remove non-specific binding of the probe. Finally, cells were pelleted down at 6000 rpm for 10 min and suspended in 200 μL PBS[5,24,25]. An aliquot of 100 μL was added to 0.5 mL of flow sheath solution (Becton Dickinson) for flow cytometry analysis.

Data acquisition by flow cytometry

Data acquisition was performed with a FACS calibur flow cytometer (Becton Dickinson) which is equipped with an air-cooled argon-ion laser providing 15 mW at 488 nm light combined with a 635 nm red-diode laser. The 488 nm laser was used to measure the forward angle light scatter (FSC, in the 488/10 nm band pass filter), the side angle light scatter (SSC, in the 488/10 nm band pass) and the green fluorescence intensity conferred by FITC labeled probes (filter 1 in the 530/30 nm band pass filter). The red-diode laser was used to detect the red fluorescence conferred by Cy5 labeled probes (filter 4 in a 661/16 nm band pass filter). The acquisition threshold was set in the side scatter channel. All the parameters were collected as logarithmic signals. The rate of events in the flow was set at low (12 μL/s). A total of 25000 events were collected and subsequent analyses were conducted using the Cell Quest Software (Becton Dickinson).

Enumeration of bacterial groups in fecal samples

Enumeration of bacterial groups was performed by a double staining method in the same reaction tube where the hybridization of the EUB338 probe labeled with FITC and the genus-specific probe labeled with Cy5 were combined. This led us to estimate the abundance of bacterial groups targeted by the respective Cy5-labeled probe as a proportion of total bacteria labeled with the EUB338 FITC probe. Next, the abundance of known butyrate producers was enumerated as cells hybridized with the FITC-labeled species-specific probe as a proportion of total cells hybridized with the respective genus-specific Cy5-labeled probe. Each time, the proportion of hybridized bacteria was corrected by subtracting the background fluorescence obtained with hybridization of the negative control probe NONEUB338.

Quantitative polymerase chain reaction

Genomic DNA from human fecal samples (220 mg) was extracted using the Qiagen stool DNA kit and eluted in 50 μL of Tris-EDTA buffer. About 20 ng of DNA from each sample was used to analyze the bacterial population. All primer sets used in the study were designed from the *16S rRNA* gene as shown in Table 2. Genus-specific primers were used to amplify respective genus and species from genomic DNA of the fecal samples of healthy individuals. The amplified product was cloned and sequenced and sequences were deposited in the EMBL database to obtain the accession numbers (Table 3). These *16S rRNA* gene fragments containing plasmids were used as reference strains. The standard curves were constructed by serial dilutions of each reference clone prepared from 0.05 to 500000 pg/tube, corresponding to 1 × 10 to 1 × 10^7 copy numbers. The standard curve of the reference clones was used to extrapolate the numbers of bacteria present in the fecal samples. With the molecular mass of the plasmid and insert known, the copy number was calculated as follows: mass in Daltons (g/molecule) = [size of double-stranded (ds) product in base pairs (bp)] (330 Da × 2 nucleotides (nt)/bp]/Avogadro's number.

Thus, the precise number of molecules (molecules/μL) = Conc./mass in Daltons[26].

Gas chromatography analysis of fecal SCFA

Fecal SCFAs were analyzed using gas chromatography/flame ionization detection (GC-FID). An aliquot of fecal

Table 2 Probes and oligonucleotides employed in the study

Primer or probe	Target (phylogenetic group)	Sequence (5′-3′) from *16S rRNA* gene	Used in FISH-flow cytometry or qPCR	Ref.
NON338	No bacteria	ACATCCTACGGGAGGC	Probe[1]	[21]
EUB338	Most bacteria	GCTGCCTCCCGTAGGAGT	Probe[1]	[20]
Erec 482	*C. coccoides*/*E.rectale* cluster	GCTTCTTAGTCARGTACCG	Probe[1]	[34]
Clep 1156	*Clostridium leptum* subgroup	GTTTTRTCAACGGCAGTC	Probe[1]	[35]
Fpra-655	*F. prausnitzii*	CGCCTACCTCTGCACTAC	Probe[1]	[16]
Rint-623	*R. intestinalis* subcluster	TTCCAATGCAGTACCGGG	Probe[1]	[16]
Ehal-057	*E. halliii* L2-7/*E. halliii*	TTGCACTGCCACCTACGC	Probe[1]	[16]
Cp1	Competitor 1	GRTTTRTCAYCGGCAGTC	Competitor[1]	[23]
Cp2	Competitor 1	GTVTTRTCBACGGCAGTC	Competitor[1]	[23]
H1174	Helper oligonucleotide	TTGACGTCRTCCCCACCTTCCTCC	Helper[1]	[23]
H1129	Helper oligonucleotide	TAGAGTGMTCTTGCGTA	Helper[1]	[23]
H1090	Helper oligonucleotide	GGTTGCGCTCGTTGCGGGACTTAA	Helper[1]	[23]
H750	Helper oligonucleotide	TCGHGCCTCAGCGTCAG	Helper[1]	[23]
H122	Helper oligonucleotide	GAAGGCAGGTTACTCACGC	Helper[1]	[23]
Clep FP	*C. leptum* subgroup	CGTCAGCTCGTGTCGTGAGAT	Primer set[2]	[36]
Clep RP		CGTCATCCCCACCTTCCTCC		
C.cocci FP	*C. coccoides* subgroup	GCCACATTGGGACTGAGA	Primer set[2]	[36]
C.cocci RP		GCTTCTTAGTCAGGTACCG		
Fpraus FP	*F. prausnitzii*	GATGGCCTCGCGTCCGATTAG	Primer set[2]	[37]
Fpraus RP		CCGAAGACCTTCTTCCTC		
Rint FP	*Roseburia*/*E. rectale* cluster	CKGCAAGTCTGATGTGAAAG	Primer set[2]	This study
Rint RP		GCGGGTCCCCGTCAATTCC		
Ehal FP	*E. hallii* L2-7/*E. hallii* members	GCGTAGGTGGCAGTGCAA	Primer set[2]	[38]
Ehal RP		GCACCGRAGCCTATACGG		

[1]Respective probe or oligonucleotide was used in fluorescent *in situ* hybridization (FISH)-flow cytometry; [2]Respective primer was used in quantitative polymerase chain reaction (qPCR). FP: Forward primer; RP: Reverse primer; *R. intestinalis*: *Roseburia intestinalis*; *E. hallii*: *Eubacterium hallii*; *F. prausnitzii*: *Fecalibacterium prausnitzii*; *C. coccoides*: *Clostridium coccoides*; *C. leptum*: *Clostridium leptum*; *E. rectale*: *Eubacterium rectale*.

Table 3 Accession number of reference strain used in the study

Bacteria	Source	Accession No.
C. leptum	Healthy human fecal sample	AM042697
F. prausnitzii	Healthy human fecal sample	JX556686
R. intestinalis	Healthy human fecal sample	JX556688
E. hallii	Healthy human fecal sample	JX556687

C. leptum: *Clostridium leptum*; *F. prausnitzii*: *Fecalibacterium prausnitzii*; *R. intestinalis*: *Roseburia intestinalis*; *E. hallii*: *Eubacterium hallii*.

content (250 mg) was extracted with 1 mL of extraction buffer [0.1% (w/v) $HgCl_2$ and 1% (v/v) H_3PO_4] supplemented with 0.045 mg/mL 2,2-dimethylbutyrate (as internal standard). The resulting slurry was centrifuged for 30 min at 5000 *g* at 4 ℃, and the supernatant was filtered through a 0.2-μm filter. SCFAs in the supernatant collected were analyzed using a GC (Shimadzu-2010) equipped with FID and a stabilwax column (Restek, United States) of 30 m length, 530 μm diameter and 1 μm film thickness. The system was run with nitrogen as carrier gas at an inlet constant pressure of 18.1 kPa. Samples were run at an initial temperature of 120 ℃ for 0.5 min, and then with 8 ℃/min change in temperature till it reached 220 ℃ and was held at 220 ℃ for 8 min for a total program time of 20.5 min[27]. SCFAs were identified using external standards consisting of acetate, *iso*-butyrate, *n*-butyrate (Sigma, India) and the concentration was calculated using the area percentage method.

Ethics statement

Ethical clearance for the study was obtained from the Institute Ethics Committee, All India Institute of Medical Sciences, New Delhi. Written informed consent was obtained from all the participants.

Statistical analysis

The mean cell proportion and number of bacteria in fecal samples were estimated by FISH and qPCR in triplicate, and the results were expressed as a percentage of bacteria and number of bacteria, respectively. SCFA level was determined by GC, and the results were expressed in mmol/μL. Student's *t* test was employed to check any significant changes in the SCFA concentrations with the changes in the disease activity. Graphpad prism-6 was used to analyze FISH and qPCR data.

RESULTS

Analysis of *C. leptum* and *C. coccoides* groups

FISH-flow cytometry: In Figure 1A, the region R1 corresponds to relative size (FSC) with granularity (SSC) of the bacteria during flow cytometry with NONEUB338 hybridized cells. This region R1 was gated for further dot plots. Flow cytometric analysis of the hybridized samples gave a shift in signal of 1 log unit compared to the nonhybridized cells, enabling the specific detection and enumeration of the different bacterial groups (Figure 1B-D). Scoring of bacteria could not be achieved uni-

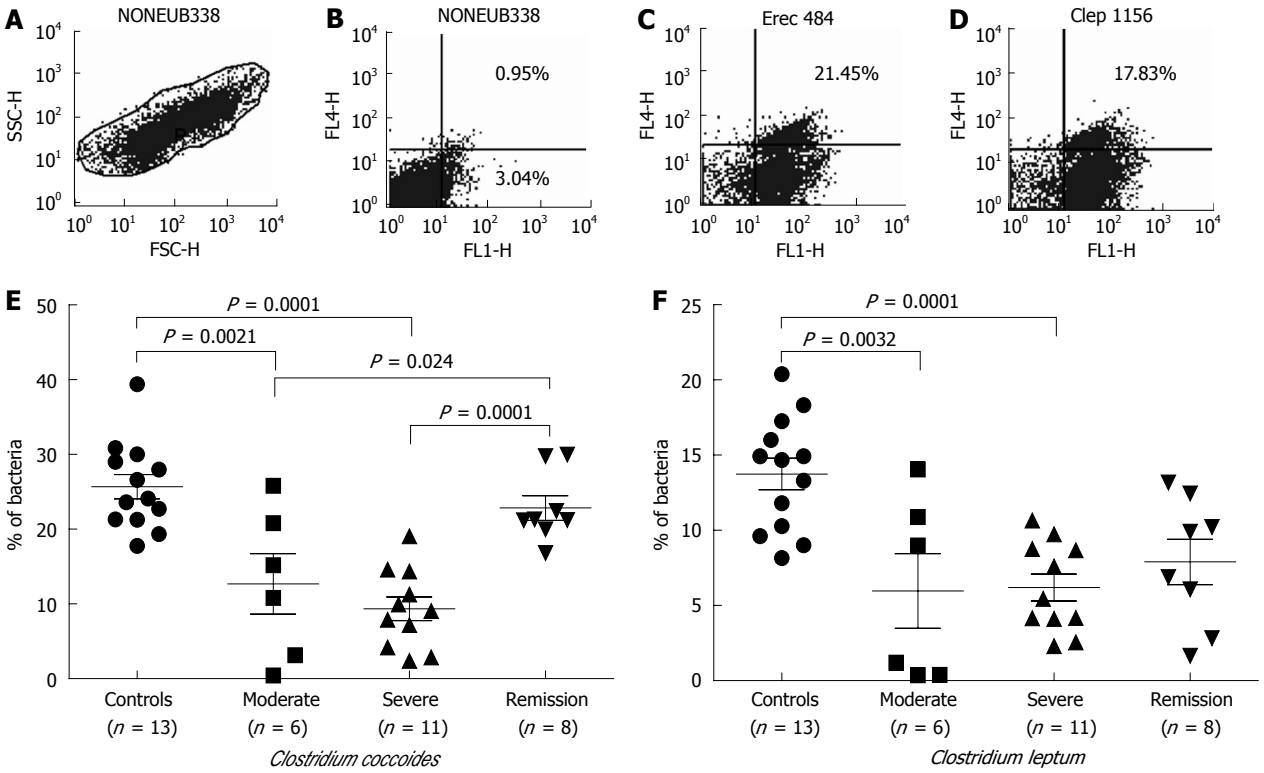

Figure 1 Flow cytometric analysis of fecal microflora using 16S rRNA targeted probes. A: The region R1 corresponding to relative size (the forward angle light scatter)/granularity (the side angle light scatter) of the bacteria was delineated. This region R1 was gated for further dot plots; B: Bacteria from fecal samples were hybridized with NONEUB338 probe; C: EUB338 and Erec 482; D: EUB338 and Clep 1156. A shift in fluorescence to higher intensities was obtained upon hybridization with positive control or group-specific probe: right lower and upper quadrant compared to the lower left quadrant. The signal in lower left quadrant represents debris. The events in upper right quadrant represent the proportion of bacterial cells hybridized with the group-specific probe within the total bacterial cells hybridized with the universal bacterial probe EUB338-fluorescein isothiocyanate (FITC). The enumeration of targeted cells was corrected by subtracting the background fluorescence, which was measured using the negative control NONEUB338 probe. Fluorescent *in situ* hybridization (FISH)-flow cytometry data were expressed as the mean % group-specific probe/EUB338 ± SE as enumerated by FISH-flow cytometry in control, moderate, severe and remission samples of ulcerative colitis; E: Erec 482; F: Clep 1156.

formly; therefore, sample size differed in each category.

In UC patients belonging to the moderate and severe disease categories, we observed significant decreases in members of the *C. coccoides* and *C. leptum* groups compared to control individuals (Figure 1E and F). Among the *C. coccoides* cluster, decreases in moderate disease samples attained the *P* value of 0.0021, while there was a *P* value of 0.0001 in the case of severe patient samples in comparison to controls. However, in the case of *C. leptum,* the *P* values were 0.0032 in moderate and 0.0001 in severe categories of samples, respectively, as compared to controls. Samples in the remission stage showed significant restoration in the population of the *C. coccoides* group ($P = 0.0001$); although an increasing trend was observed in the members of the *C. leptum* group, this did not attain a significant value.

Quantitative polymerase chain reaction: In order to validate our FISH-flow cytometry data, we carried out a qPCR study (Figure 2A and B), where we observed significant decreases in both the members of *C. coccoides group* ($P = 0.027$) and *C. leptum* group ($P = 0.041$) in samples of moderate and severe disease stages. Members of both the clusters showed restoration of bacteria in samples of remission category; however, this did not attain a significant *P* value.

SCFA quantification by GC: We further quantified the change in concentration of fecal SCFAs, namely acetate, *n*-butyrate and *iso*-butyrate, in control *vs* UC patient samples by GC. The concentrations of butyrate ($P = 0.003$), *iso*-butyrate ($P = 0.044$) and acetate ($P = 0.047$), were significantly reduced in severe UC samples when compared with control samples (Figure 3). As expected, during the remission stage *n*-butyrate level significantly restored back to normal level ($P = 0.05$) as seen in control individuals, confirming that the decrease observed during disease conditions reflects the loss of butyrate-producing bacteria (Figure 3C).

Evaluation of predominant butyrate producers

FISH-flow cytometry: Next, we evaluated the concentration of predominant members of both clostridial clusters (XIVa and IV). The population of *F. prausnitzii,* a member of the *C. leptum* group, was significantly low in UC samples of severe ($P = 0.0001$) category of disease in comparison to control samples (Figure 4A). Samples from the remission stage did not show significant restora-

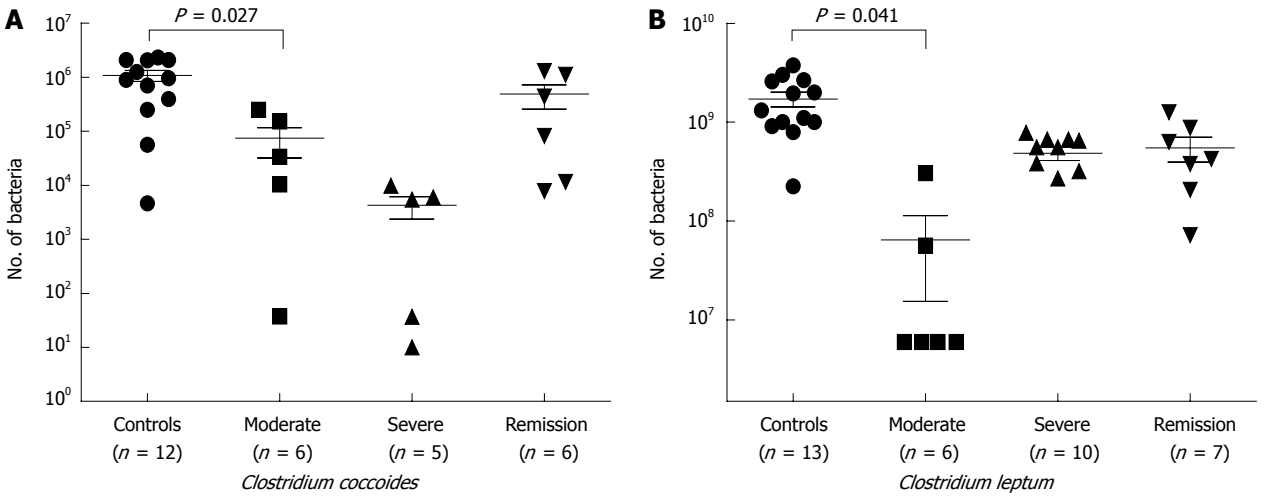

Figure 2 Quantitative polymerase chain reaction data showing the numbers of bacteria (± SE) in fecal samples of control *vs* ulcerative colitis patients. A: *Clostridium coccoides;* B: *Clostridium leptum*. The *Y* axis represents number of bacteria and *X* axis represents the sample category.

tion of *F. prausnitzii* in our FISH-flow cytometry experiment, as was observed for the *C. leptum* cluster during remission (Figures 1F and 2B).

R. intestinalis, a member of the *C. coccoides* group, was also significantly low (P = 0.02) in patient samples of severe category disease (Figure 4B); however, this did not show significant restoration in the samples of remission stage. The population of *E. halii,* another member of the *C. coccoides* group, decreased in disease conditions, but not significantly (Figure 4C). We failed to detect *E. hallii* in the majority of samples either by flow cytometry or qPCR, indicating low abundance of these bacteria in our study population. An increase in the population of *R. intestinalis* was recorded during remission stage in comparison to severe stage of the disease (Figure 4B and C). This was in agreement with our FISH-flow cytometry data of *C. coccoides* group. Abundance of *R. intestinalis, E. hallii* and *F. prausnitzii* was calculated out of total microbiota, as shown in Table 4. We detected a higher representation of *R. intestinalis* compared to *E. hallii* and *F. prausnitzii*. Therefore, we can infer that at the species level, abundance of the members of the *C. coccoides* cluster was higher in comparison to the members of the *C. leptum* cluster.

Quantitative polymerase chain reaction

As expected, the population of *F. prausnitzii* was significantly reduced in the samples of severe category of UC disease (P = 0.045) (Figure 4D) as compared to control. In addition, abundance of the same species was restored at remission stage when compared to severe stage (P = 0.041) (Figure 4D). Similarly, the *R. intestinalis* population showed significant reduction in samples of moderate (P = 0.015) and severe (P = 0.001) stage of the disease in comparison to control (Figure 4E). The recovery in the population of *R. intestinalis* was also seen in the remission category of samples when compared to severe category (P = 0.018) (Figure 4E). However, qPCR analysis in the case of *E. hallii* did not show any significant reduction; in addition, *E. hallii* was undetected in the majority of samples observed by FISH-flow cytometry (Figure 4F).

DISCUSSION

Our study revealed significant reduction in the members of both *C. coccoides* and *C. leptum* groups in fecal samples obtained from the severe disease category of UC patients in comparison to controls. FISH-flow cytometry and qPCR analysis of fecal samples belonging to the above groups supported the observations made by Takaishi *et al*[4] and Sokol *et al*[5].

We quantified the abundance of predominant butyrate-producing species of clostridial clusters to see their association with acetate and butyrate. Since in most cases butyryl-CoA:acetate CoA-transferase rather than butyrate kinase appears to perform the final step in butyrate synthesis, we targeted bacteria possessing butyryl CoA:acetate CoA-transferase for butyrate synthesis, *e.g.*, *F. prausnitzii* and *Roseburia spp*/*E. rectale* which apparently lack butyrate kinase activity[15,28]. It is known that *Roseburia spp.* and *F. prausnitzii* strains contribute in butyrate production and many strains are associated with the net consumption of both acetate and carbohydrate[15]. Our study revealed that both the above species exhibited low abundance in the samples of severe category of UC and tended to restore their population during remission.

F. prausnitzii has been shown to exhibit anti-inflammatory effects on cellular and trinitrobenzenesulphonic acid-induced colitis models, partly due to secreted metabolites that are able to block NF-κB activation and interleukin 8 production[29]. In our observations (Table 4), *F. prausnitzii* accounted for 11.66% ± 1.55% of the *C. leptum* group, and *R. intestinalis* and *E. hallii* accounted for 14.48% ± 1.52% and 5.93% ± 0.54% of the *C. coccoides* group, respectively, in control individuals. In total these three species accounted for 6.83% of the total fecal flora. Previous studies have already reported low counts of *F. prausnitzii* in UC patients[30].

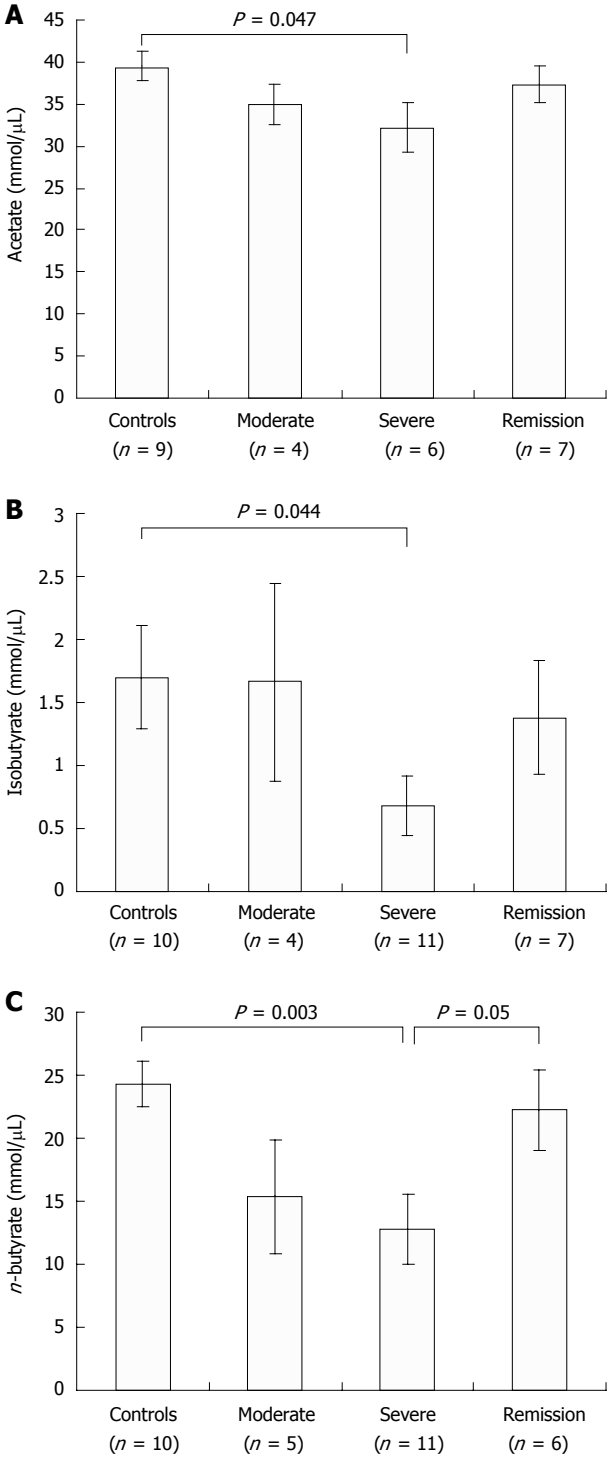

Figure 3 Short chain fatty acids in fecal samples of ulcerative colitis patients *vs* control samples, analyzed by gas chromatography. A: Acetate; B: *Iso*-butyrate; C: Butyrate. The *Y* axis represents concentration of respective short chain fatty acid and *X* axis represents the sample category.

We observed a significant decrease in concentrations of butyrate, *iso*-butyrate and acetate in UC fecal samples compared to controls through SCFA quantification by GC analysis, supporting earlier observations[4,31].

An intriguing link between the level of SCFA and an intracellular energy sensor for the maintenance of intestinal barrier function has been suggested[10]. Our study further substantiates a link between the fluctuations of butyrate production and the changes in the numbers of butyrate producers during UC. Lack of butyrate availability may lead to compromised intestinal barrier function resulting in increased exposure of luminal content to the host immune system, thus exaggerating the immune response[10].

The reduced population of dominant butyrate producers and decreases in concentrations of butyrate and acetate in diseased samples indicate impaired butyrate supply in the colon, which may lead to energy deficiency for colonocytes. The resurgence of butyrate-producing bacteria at the remission stage and simultaneous increases in butyrate concentration, as observed here, show an association with UC and support the energy deficiency hypothesis of IBD[32]. Earlier studies have shown a requirement of acetate by *F. prausnitzii* and *R. intestinalis* for survival and other activities like production of butyrate[15,33]. Thus, major butyrogenic species depend on other bacteria, including net producers of acetate (*e.g.*, amylolytic bifidobacteria) and other bacterial species capable of degrading a variety of complex carbohydrates[15]. These bacteria play an important role in net butyrate production and may be associated with dysbiosis during UC.

Acetate and butyrate are major SCFAs and decreases in their concentration may affect the overall SCFA concentration. SCFAs in the colon maintain mild acidic conditions which boost the formation of butyrate by favoring growth of butyrate-producing bacteria and allowing them to compete against gram-negative bacteria such as *Bacteroides spp* to maintain the homeostasis[9]. Thus, a reduced butyrate level may lead to an increased Gram-negative bacteria population due to reduced competition. This was validated by our observation of increased representation of *Bacteroides* in UC samples (data not shown).

Our data showing reduced abundance of three predominant butyrate-producing species indicate that during UC, butyrate deficiency may not be solely due to reduced uptake of butyrate by the inflamed mucosa as reported by Canani *et al*[9], but also due to reduced abundance of predominant butyrate producers and conversely lower production of butyrate.

The limitation of our findings is that most of the microbial data are not presented in absolute values but in percentages; therefore, it is difficult to critically assess the real changes in the microbial composition. The study is performed in a very restricted number of subjects due to the exclusion criteria followed during sample collection.

In conclusion, our study shows a decreased abundance of predominant butyrate producers like *R. intestinalis* and *F. prausnitzii* belonging to clostridial clusters in the UC disease condition. This decrease was found to correlate with reduced SCFA concentration in UC patients. We thus provide evidence that reduced butyrate levels during the diseased state are due to less abundance of these species in UC. It is evident from our data that decreased levels of these species resulting in reduced butyrate levels may be associated with the etiology of UC.

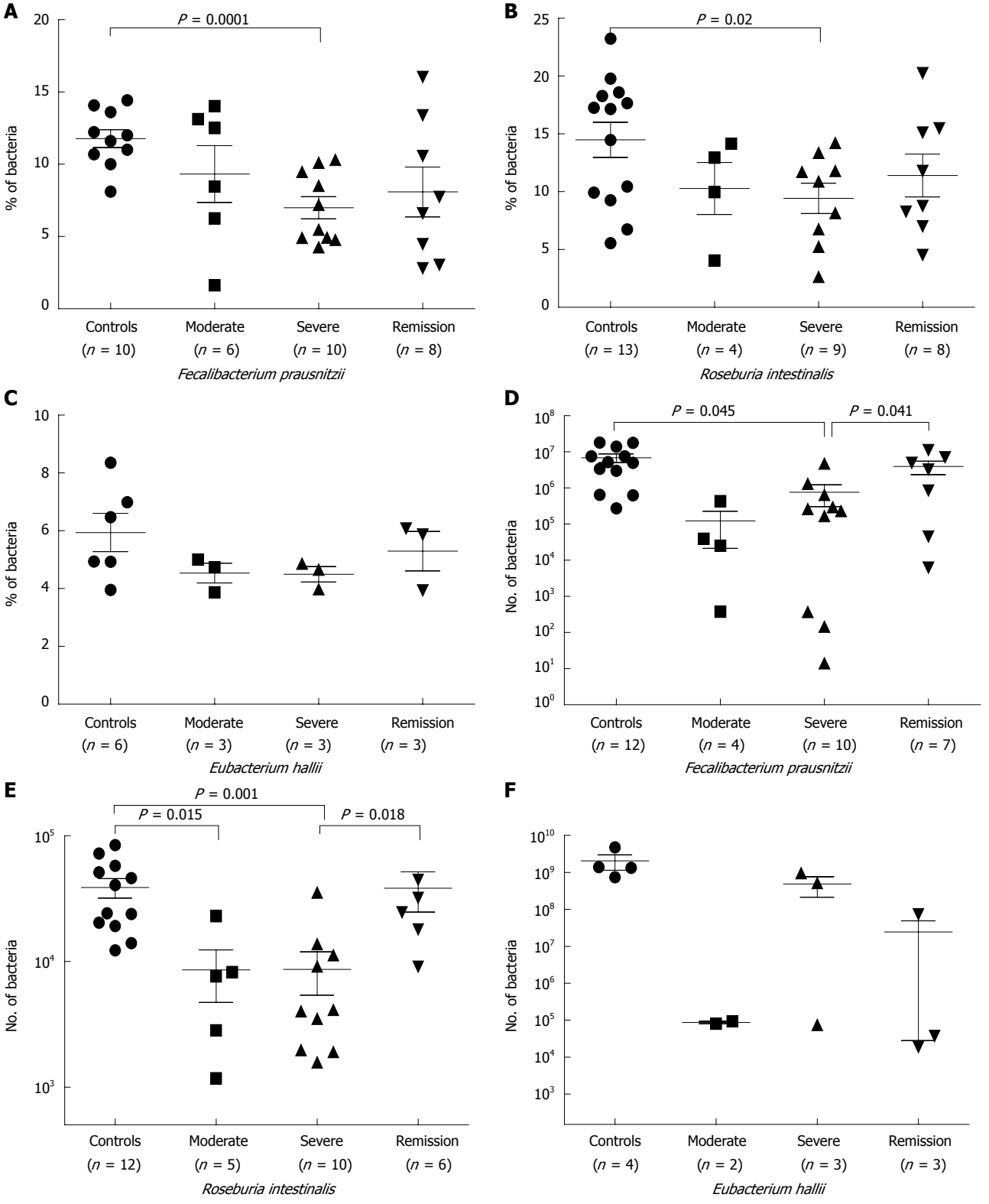

Figure 4 Analysis of predominant butyrate-producing bacteria. A-C: *Fecalibacterium prausnitzii* (A), *Roseburia intestinalis* (B), and *Eubacterium rectale* (C) by fluorescent *in situ* hybridization-flow cytometry; D-F: *Fecalibacterium prausnitzii* (D), *Roseburia intestinalis* (E), and *Eubacterium hallii* (F) by quantitative polymerase chain reaction. The *Y* axis represents number of bacteria and *X* axis represents the status of patients. Horizontal bars with asterisks represent comparison between ulcerative colitis and control conditions.

This was further demonstrated in our analysis of the samples from remission patients, where the targeted organisms tended to revert back to normal level. It would be of interest in the future to extend this approach to studying the diversity of each member of the clostridial cluster, including changes in non-butyrate producers.

Table 4 Enumeration of butyrate producers in fecal samples through fluorescent *in situ* hybridization-flow cytometry (mean ± SE)

Cluster or species	Out of total microbiota	Species	Out of respective cluster
Control fecal samples			
C. coccoides cluster	25.69% ± 1.62%	*R. intestinalis*	14.48% ± 1.52%
		E. hallii	5.93% ± 0.54%
C. leptum cluster	13.74% ± 1.05%	*F. prausnitzii*	11.66% ± 1.55%
R .intestinalis[1]	3.71% ± 0.39%		
E. hallii[1]	1.52% ± 0.13%		
F. prausnitzii[1]	1.60% ± 0.21%		
UC fecal samples (moderate)			
C. coccoides cluster	12.70% ± 4.60%	*R. intestinalis*	10.00% ± 2.25%
		E. hallii	4.03% ± 0.3%
C. leptum cluster	6.40% ± 3.40%	*F. prausnitzii*	7.5% ± 2.3%
R .intestinalis[1]	1.27% ± 0.10%		
E. hallii[1]	0.51% ± 0.01%		
F. prausnitzii[1]	0.48% ± 0.14%		
UC fecal samples (severe)			
C. coccoides cluster	9.80% ± 2.40%	*R. intestinalis*	9% ± 1.83%
		E. hallii	4.2% ± 0.2%
C. leptum cluster	6.20% ± 1.80%	*F. prausnitzii*	6.01% ± 1.6%
R .intestinalis[1]	0.82% ± 0.04%		
E. hallii[1]	0.41% ± 0.01%		
F. prausnitzii[1]	0.37% ± 0.02%		
UC fecal samples (remission)			
C. coccoides cluster	12.99% ± 2.65%	*R. intestinalis*	11.40% ± 1.83%
		E. hallii	5.07% ± 0.6%
C. leptum cluster	7.89% ± 1.50%	*F. prausnitzii*	7.40% ± 2.32%
R .intestinalis[1]	1.48% ± 0.04%		
E. hallii[1]	0.65% ± 0.01%		
F. prausnitzii[1]	0.58% ± 0.03%		

[1]Abundance of *Roseburia intestinalis* (*R. intestinalis*), *Eubacterium hallii* (*E. hallii*) and *Fecalibacterium prausnitzii* (*F. prausnitzii*) were calculated out of total microbiota. *Clostridium coccoides* (*C. coccoides*) and *Clostridium leptum* (*C. leptum*) were enumerated as proportion of total bacterial cell hybridized with group-specific probe. *R. intestinalis, E. hallii* and *F. prausnitzii* were enumerated as proportion of cell hybridized with species-specific probe. UC: Ulcerative colitis.

COMMENTS

Background

Inflammatory bowel disease (IBD), comprising of Crohn's disease and ulcerative colitis (UC), is a class of chronic inflammatory disorders of the intestine and involves impaired barrier function. A rising trend in the incidence and prevalence of inflammatory bowel IBD in the Asian population has been recognized for the past two decades. IBD is considered as an exaggerated immune response, exerted either to all commensal bacteria or to a subset or to a single strain of bacteria.

Research frontiers

Differences in fecal microbiota of healthy subjects and IBD patients have been identified. Commensal gut microbiota have been reported to exert various multiple beneficial effects on host gut epithelium. short chain fatty acids (SCFAs), like butyrate, produced by microbial fermentation of undigested carbohydrates have been depicted to regulate transepithelial transport, colonocyte proliferation and differentiation, mucosal inflammation, intestinal motility and barrier function. The homeostasis between commensal micobiota and host defensive responses at the mucosal level has a pivotal role in the initiation and pathophysiology of chronic IBD. Therefore, monitoring the fluctuations in butyrogenic bacteria and SCFA level may help in understanding the relation of dysbiosis with UC.

Innovations and breakthroughs

This work was carried out to study the fluctuations in butyrate-producing bacteria and simultaneous measurement of butyrate concentration in fecal samples of UC *vs* control individuals belonging to the northern part of India. The data were also correlated with patients differing in disease activity. Decreases in the level of butyrate producers in UC patients coincided with the changes in butyrate concentration during active stage disease. However, the levels reverted back to normal during remission stage showing the role of these bacteria in the disease etiology. Such comparisons have not been made so far to show the above relationship with disease activity.

Applications

It is speculated that by measuring the level of these species and concentration of SCFA in stool samples (a non-invasive method), authors can indicate the status of UC patients.

Terminology

Dysbiosis (also called dysbacteriosis) refers to a condition with microbial imbalances on or within the body. Dysbiosis is most prominent in the digestive tract or on the skin, but can also occur on any exposed surface or mucous membrane such as the vagina, lungs, mouth, nose, sinuses, ears, nails or eyes. It has been associated with different illnesses, such as IBD, as imbalances in the intestinal microbiome may be associated with bowel inflammation and chronic fatigue syndrome.

Peer review

This is a methodologically well performed, interesting report, addressing the potential role of gut commensal microbiota in the pathophysiology of UC.

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