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Original article

Altered fecal bacterial composition correlates with disease activity in inflammatory bowel disease and the extent of *IL8* induction

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ABSTRACT

Purpose of the study: In this study we investigated the presence and relative abundance of important genera of the gut microbiota in IBD patients and their role in induction of *IL8* in a cell culture model.
Patients and methods: Stool samples of IBD patients and healthy controls were collected and relative diversity of thirteen bacterial families was measured using quantitative real-time PCR assay. Moreover, filtrate of the stool samples was used for treatment of HT-29 cell line to analyze involvement of diversity of the fecal bacterial communities in the extent of *IL8* induction.
Results: *Bacteroides, Faecalibacterium prausnitzii, Prevotella* spp., and *Methanobrevibacterium* were significantly less abundant in IBD patients (UC, N = 22; CD, N = 7) compared with control group (N = 29). Increase in relative amounts of *Haemophilus*,*Streptococcus* spp., and *H. pylori* were detected in IBD patients, which was not statistically significant. Relative decrease in amount of *Bacteroides* spp., *Faecalibacterium prausnitzii*, and *Prevotella* spp. were found in UC patients with disease activity score greater than 4; however, higher levels of *Streptococcus* and *Haemophilus* were detected in the patients who were at flares. A relationship between the reduction of *Haemophilus* spp. and higher BMI was shown in IBD patients. Expression of *IL8* was significantly higher in the treated cells by the fecal inoculates of IBD patients. Increase in relative amounts of *Enterobacteriacea* showed a correlation with the higher level of *IL8* induction in both groups.
Conclusions: These results showed that changes in the fecal microbiota composition could affect disease activity, BMI, and *IL8* induction.

Introduction

Inflammatory bowel disease (IBD) includes various forms of disorders; however Crohn's disease (CD) and ulcerative colitis (UC) are two major types of this disease. Ulcerative colitis is characterized by mucosal inflammation in the colon, while Crohn's disease is characterized by patchy, transmural inflammation, that may affect all parts of the gastrointestinal tract [1]. There is some evidence that show role of genetic and immune disorders and environmental factors, such as change in fecal microbiota, in the pathogenesis of IBD [2,3].

Different families of bacteria showed correlation with IBD; however it is not well known whether they are innocent bystander or play roles in the occurrence or exacerbation of the disease and

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other systematic disorders in these patients. Changes in the composition of the mucosa associated bacteria in the intestine, which is known as dysbiosis, could promote IBD through deregulated induction of immune system, genomic instability, and induction of mutations. Alternatively, dysbiosis could cause to dysfunction of innate immune system in the intestine, or induction of local immune system, such as higher colon tissue IL-8 and IL-6 levels, in these patients during active phase of the disease, which may explain its role in the disease activity [4]. These interplay could be induced by direct interaction of the bacteria or indirectly through their metabolites and toxins [5,6]. There is growing evidence that exhibited involvement of these bacteria in some other diseases, such as development of obesity and metabolic disorders, e.g type 2 diabetes and non-alcoholic fatty liver disease [NAFLD] [7–10].

Molecular studies showed that patients with UC present a moderate degree of gut microbial dysbiosis, a decrease in the abundance of some universal bacteria, *e.g. Bacteroides* and *Firmicutes*, and an increase in various opportunistic pathogens, as well as an enrichment of other microbial families, such as *Gammaproteobacteria* [11]. However, interpretation of these changes is difficult, because of the differences in the study populations, types of samples and molecular techniques that were used. While relationship between exacerbated inflammation in the intestine of IBD patients and resident microbial populations is unknown, this relationship was shown for some other inflammatory diseases such as rheumatoid arthritis [12]. Recent studies showed a greater risk for IBD onset and relapse through infection with some enteric pathogens, such as adherent-invasive *Escherichia coli*, *Salmonella*, and *Clostridium difficile* [13]; however this link needs to be proven, since, administration of immunosuppressants after onset of the disease could also promote infection with these pathogens [14–16]. There are spare investigations on effects of anti-inflammatory drugs that commonly prescribed for IBD treatments on the gut microbiota. Adverse effects of mesalamine and 5-aminosalicylic acid on abundance of members of *Enterobacteriacea*, *Enterococcus* and *Faecalibacterium* spp. were described in some studies [15,17,18]. Antibiotic usage is among main risk factors that can affect microbial composition of the intestine and are associated with elicit of IBD, mainly CD [19,20]. Whether these medications affect disease severity and are associated with disease flares are not well known [21,22].

Despite recent progress in detection of common bacterial communities in IBD patients, no definite microbial agents and related mechanisms of pathogenesis in disease onset and its progression have been described. To address this issue, diversity and relative abundance of most commonly associated bacterial families with IBD was investigated in our patients compared with healthy controls. Furthermore, diverse effects of fecal bacterial compositions on extent of IL8 induction were analyzed in a cell culture method. Possible associations among treatment regimens, composition of the bacterial families, body mass index, and severity of the diseases were also determined in the patients compared with healthy controls.

Materials and methods

Study design and sample collection

To investigate diversity of fecal microbiota, freshly prepared fecal samples were obtained from IBD patients and healthy volunteers in Ayatollah Taleghani Hospital, Tehran, Iran. All the IBD patients were characterized by both colonoscopic and pathologic criteria in the gastroenterology unit. Healthy volunteers, who presented no recent history of antibiotic usage and medications (within the past three months), community acquired diarrhea (within the last month), hospitalization (within the past 12 months), recent gastrointestinal surgery and illness, or specific dietary regimens, were randomly selected from same age groups during the study period. Informed consent forms were obtained from the patients and healthy volunteers according to a protocol approved by the ethical review committee of Shahid Beheshti University of Medical Sciences, Tehran, Iran. Demographic features were recorded for all the participants. To measure disease activity of UC and CD, criteria that were proposed by Sutherland LR. et al. and Best WR. et al. were used, respectively [23,24]. Demographic data were collected by questionnaire to provide data about underlying diseases, medication and antibiotic usage, surgery of GIT, gender, and age. Patients who received antibiotics at time of sampling, those who presented recent history of antibacterial medication (within the past three months), community acquired diarrhea (within the last month), and/or surgery, were excluded from the study.

All the samples were collected in a clean container and transferred to the laboratory during one hour. Total DNA was extracted from the fecal samples using DNA extraction kit (Bioneer, South Korea) and the DNA samples were stored at −20 °C until use for further investigations.

Furthermore, 3 g of the fresh fecal samples were homogenized with 5 ml sterile normal saline. The samples were filtered through Whatman filter paper at sterilized conditions to remove debris. Portion of each filtrate was mixed with glycerol at 20% final concentration and were kept at −70 °C for cell culture experiment.

Relative abundance of microbiota using quantitative real-time polymerase chain reaction

Thirteen bacterial families/genera, with statistically significant relative increase or decrease in the feces of IBD (UC and CD) patients compared with controls, were considered from the studies in other countries. These bacteria showed greatest association with the disease. Primer sets suitable for real time PCR were selected for bacterial genera to compare the abundance of these bacteria in IBD patients and healthy controls [25–39]. Name of the bacteria and related oligonucleotide primers used in this study are listed in Table 1. Total concentration of DNA extracts were adjusted before performance of the assay. To prevent formation of primer dimer and non-specific products during real time PCR, melt curve analysis and PCR were done. Amplification of target sequences for each bacteria and total bacteria was done in separate reactions in each 96-well plate read using Applied Biosystems 7500 Real-Time PCR System. The ΔΔCT method of comparison was used to compare relative abundance of bacterial species in each sample ($2^{-\Delta CT} = 2^{-(CT\ target\ bacteria\ in\ each\ sample\ -\ CT\ target\ bacteria\ in\ reference\ sample)}$). DNA extract of a randomly selected control sample was used as calibrator (reference sample) in all the experiments. Two separate replica of the assay were performed for all the experiments and average amount of threshold cycles (C_T) of two separate examinations for each bacterium was used for the calculation. This amount was presented as fold change for all the bacteria. Any increase or decrease in the population of bacterial families was determined based on cut off values (mean RQ ± 2 SD for each bacterial family) measured in the healthy control group.

PCR and real time PCR conditions for bacteria

To evaluate quality of the primers for identification of target genes in each bacterial genus, PCR was done. The 25 μl PCR reaction mixture contained 2.5 μl of 10X PCR buffer, 1.25 μl of each primer (10 picomol), 0.5 μL of each deoxyribonucleotide triphosphate (dNTP, 10 mM), 0.75 μl $MgCl_2$ (50 mM), 0.75 U of TaqDNA polymerase (5 U/ml) and 1 μL of DNA template from

Table 1
Oligonucleotide primer sequences that were used in this study.

Baceria	Oligonucleotide sequence (5′→3′)	Amplicon size	Reference
Methanobrevibacter	F: CGATGCGGACTTGGTGTTG R: TGTCGCCTCTGGTGAGATGTC	183 bp	[25]
Bacteroides	F: GGGGTTCTGAGAGGAAG R: GCTACTTGGCTGGTTCAG	115 bp	[25]
Clostridium difficile	F: TTGAGCGATTTACTTCGGTAAAGA R: TGTACTGGCTCACCTTTGATATTCA	113 bp	[26]
Mycobacterium avium paratuberculosis	F: TGGTCGTCTGCTGGGTTGA R: TGCCACAACCACCTCCGTA	54 bp	[27]
Campylobacter spp.	F: CACGTGCTACAATGGCATAT R: GGCTTCATGCTCTCGAGTT	106 bp	[28]
Prevotella spp.	F: CCAGCCAAGTAGCGTGCA R: TGGACCTTCCGTATTACCGC	150 bp	[29]
Peptostreptococcus spp	F: ATAGGAGGAAGCCCTGGCTAAA R: CTCCACGCTTTGACACCTGA	134 bp	[30]
Enterobacteriaceae	F: TGCCGTAACTTCGGGAGAAGGCA R: TCAAGGACCAGTGTTCAGTGTC	428 bp	[31]
Helicobacter pylori	F: GAAGATAATGACGGTATCTAACGAATAA R: CATAGGATTTCACACCTGACTGACTAT	144 bp	[32]
Haemophilus	F: AATGGCGTATACAGAGGGAAG R: CAATCCGGACTTAGACGTACT	71 bp	[33]
Faecalibacterium prausnitzii	F: GATGGCCTCGCGTCCGATTAG R: CCGAAGACCTTCTTCCTCC	199 bp	[34]
Serratia marcescens	F: AGTGCACGAACAAACTTACAG R: GTCGTACTCGAAATCGGTCACA	138 bp	[35]
Klebsiella pneumoniae	F: ACGGCCGAATATGACGAATTC R: AGAGTGATCTGCTCATGAA	68 bp	[36]
Universal primers	F: AGMGTTYGATYMTGGCTCAG R:GCTGCCTCCCGTAGGAGT	314-373 bp	[37]
IL8	F: AGCACTCCTTGGCAAAACTG R: CGGAAGGAACCATCTCACTG	112 bp	[38]
ACTB	F: ATGTGGCCGAGGACTTTGATT R: AGTGGGGTGGCTTTTAGGATG	132 bp	[39]

pure cultures of the reference strains. It was performed in a thermal cycler (AG 22331; Eppendorf, Hamburg, Germany). A reaction with no template DNA was included as control (NTC) reaction for each bacterium. The PCR conditions was as follow: one cycle of initial denaturation at 93 °C for 1 min, 35 cycles of denaturation at 95 °C for 45 s, annealing at 62 °C for 30 s, and extension at 72 °C for 35 s. Final extension was performed at 72 °C for 10 min. PCR products were analyzed by electrophoresis in a 1.2% agarose gel after staining using ethidium bromide (0.5 μg/ml). Differences of the primers in amplification were calculated by band volume analysis using UVIgeltec software (version 12.1).

The real time PCR for relative quantification of target bacterial genera was performed in a total volume of 20 μl using 10 μl SYBR® Premix Ex TaqTM master mix (TaKaRa, Japan), 0.4 μl of each forward and reverse primer, 0.08 μl diluted ROX and 2 μl of DNA template under following conditions: one step of initial denaturation at 95 °C for 30 s, and 35 cycle repeats of denaturation at 95 °C for 5 s, annealing at 57–62 °C for 34 s, and extension at 60 °C for 34 s. The reaction was ended after final extension at 75 °C for 15 s. To analyze formation of primer dimmer or non-specific products during the amplification, melt curve analysis was performed for each primer. Accordingly, at the end step of the amplification, the reaction was continued by heating at 95 °C for 15 sond, a temperature gradient of 60–95 °C for 1 min, and 95 °C for 15 sond.

Cell culture

To measure differences in the extent of *IL8* induction of the prepared fecal filtrates of IBD patients and controls, we used human colon adenocarcinoma cell lines HT-29 that was purchased from Pasteur Institute of Iran. HT-29 cells were cultured in high glucose Dulbecco's modified Eagle's minimal essential medium (H-DMEM, Sigma, USA) supplemented with 5% (v/v) heat inactivated fetal bovine serum (FBS, Sigma-Aldrich, USA), penicillin (100 U/ml, Sigma-Aldrich, USA) and streptomycin (100 U/ml, Sigma, USA) in 25 cm^2 culture flask, and incubated in a CO_2 incubator (5% CO_2) at 37 °C. The cultures were fed with fresh medium every alternate day until the cells reach 80% confluence. Subculture of the cells was carried out in twelve-well tissue-culture plates using 0.25% Trypsin–EDTA solution after washing the confluent monolayer with phosphate buffered saline (PBS). Incubation of the cells was continued for 14-days after confluency to allow their differentiation into functional enterocytes. Cell viability was assessed by trypan blue test. Accordingly, the trypsinized cell suspension was diluted in 1:10 ratio using a 0.025% (w/v) trypan blue solution (Gibco, USA). Cell count in suspension were measured with Neubauer Improved Heamocytometer, perci color HBG (Germany).

Treatment of HT-29 cells with the stool samples

The 14-days differentiated HT-29 cells were treated with 1:10 dilution of Whatman filterated homogenized stool samples and incubated in fresh medium without antibiotics for 3 h. The treated cells trypsinized with 0.25% trypsin–EDTA solution (Sigma, USA) after three washes with PBS. Cell pellets were prepared by centrifugation at 10,000 RPM for 5 min. Total RNA was extracted using system SV Total RNA Isolation kit (Promega, USA). Concentration of the RNA extracts from each sample was quantified by Nanodrop. After adjusting the RNA amounts, cDNA synthesis was performed by cDNA synthesis kit (Prime Script TM RT reagent Kit (Takara, Japan) according to the manufacturer's instructions. For confirmation of cDNA synthesis, presence of *IL8* and *ACTB* genes was demonstrated by PCR using specific primers.

Quantitative real time PCR

To measure differences in the extent of *IL8* induction by the fecal filtrates of IBD patients and controls, relative fold changes of *IL8* transcription were determined among the treated cells using real-time PCR. This assay was performed using SYBR Green real time PCR Master Mix for 40 cycles. Real time PCR reactions were set up with specific primers for *IL8* and *ACTB* genes in 20 μl reaction mixture containing 10 μl of SYBR Green PCR Master Mix (Takara, Japan), 2 μl of cDNA, 0.4 μl of each primer (*IL8* and *ACTB* that was shown in Table 1) and 0.08 μl of 1:10 dilution of ROX. The amplification conditions were as follows: initial activation at 95 °C for 10 min, followed by denaturation at 95 °C for 15 sond; annealing and extension at 60 °C for 1 min. Melt curve analysis was performed to determine probable production of non-specific PCR products due to primer dimer formation or non-specific amplification.

Statistical analysis

Statistical difference between the patients and control groups was determined by GraphPad Prism software version 5.04 using unpaired t-test. Receiver-Operator Characteristic (ROC) curve was drawn to show sensitivity and specificity of the test for differentiation of IBD patients from control people. In the case of IL8, RQ values of less than 0.5 fold change were considered as decreased expression and those higher than 2 fold increase were defined as increased expression. Principal Component Analysis (PCA) plots and related heatmaps were drawn to visualize clustering of multivariate data.

Ethical considerations

This study was approved in Research Center for Gastroenterology and Liver Disease Shaheed Beheshti University of Medical Sciences with ethical consideration code 841.

Table 2
Comparison of demographic data in IBD patients at two different stages of the disease.

	Crohn's Disease (N=7)		Ulcerative colitis (N=22)		Control	*p* value
	(Remission	(Flare up)	(Remission)	(Flare up)		
Gender						
Male	1/3	2/3	3/10	7/10	20	
Female	3/4	1/4	6/12	6/12	9	
Age (years)						
10–20	–	–	–	–	–	
20–40	3/5	2/5	6/16	10/16	15	
41–60	1/2	1/2	3/6	3/6	14	
>60	–	–	–	–	2	
BMI[a]						**0.001**
Overweight	1/2	1/2	3/6	3/6	6	
Obesity	1/1	0	1/4	3/4	2	
Normal	1/2	1/2	5/11	6/11	21	
Underweight	1/2	1/2	0	1/1		
Disease duration						
≤6 months	–	–	–	1/1		
7–12 months	–	1/1	1/4	5/4		
1–5 years	1/1	–	4/9	5/9		
>5 years	3/5	2/5	4/8	4/8		
Drug usage						
Aminosalicylates						
Mezalazine	1/3	2/3	6/16	10/16		
Asacol	4/5	1/5	6/15	9/15		
Pentasa	1/3	2/3	1/6	5/6		
5-ASA[b]	3/6	3/6	9/22	13/22		
Sulfasalazine	2/2	–	1/4	3/4		
Immunomodulators						
Azathioprine	4/6	2/6	5/13	8/13		
Cyclosporine	1/1	–	–	–		
Corticosteroids						
Hydrocortizone	2/4	2/4	3/7	4/7		
Prednizolone	3/5	2/5	5/12	7/12		
Cortenema	–	1/1	1/2	1/2		
Anti-TNF[c]						
Infliximab	3/3	–	2/6	4/6		
Activity index						**0.0004**
0-2	NA	NA	8/9	1/9		
4- >12	NA	NA	1/12	11/12		
Polyp	–	–	–	–		
Atrophy	1/1	–	1/1	–		
Dysplasia	–	–	–	–		
Abscess	2/4	0/3	5/9	5/13		

[a] BMI, body mass index; 5-ASA.
[b] 5-aminosalicylic acid.
[c] TNF, tumor necrosis factor.

Results

Patients and sampling

A total of 29 fecal samples from IBD patients (22 with UC and 7 with CD) and 29 healthy volunteers, which show no history of other gastrointestinal disorders, were collected in this study. Among the patients with IBD, 13 patients were in the clinical remission and 16 in the flare up stages. In the IBD group, the median age was 35 years (22 to 61 years), while mean age of 31 years old was measured in the control group. Association between the patients' demographic data and disease severity was shown in Table 2. While normal weight was significantly observed among patients with UC, no significant differences were detected between disease stage (remission and flare up) and other demographic variable. Most of the UC patients who were at flare stage showed significantly higher activity index compared with those at remission stage.

Characterization of the bacteria

Results of conventional PCR showed usefulness of the primers for detection of each bacterium in the pure cultures of reference strains and in DNA extracts of the fecal samples. Melt curve analysis results refused formation of any non-specific amplicon or primer dimer for each primer during the assay.

Frequency of bacterial strains in fecal samples of IBD patients and healthy volunteers

Analysis of the prevalence of the studied bacteria showed that most of the bacterial families had similar frequency in both IBD and control groups. *Methanobrevibacter* was more frequent in healthy group (20%) compared with IBD patients, 4% and 0% in UC and CD patients, respectively. Frequency of *Peptostreptococcus* spp. showed no difference in the controls (10%) compared with the UC patients (9%). While none of the CD patients carried *Clostridium difficile*, a similar frequency of the infection was detected in UC patients and control groups (3%). Generally, among the bacterial strains that examined in this study, *Serratia marcescens, Mycobacterium avium* sub species *paratuberculosis* and *Campylobacter* spp. were not detected in fecal samples of both groups. *Klebsiella pneumoniae* was detected in 3% of IBD patients (one patient with CD), while no infection was confirmed among the healthy controls. *Faecalibacterium prausnitzii, Haemophilus, Helicobacter pylori, Bacteroides, Prevotella* spp. and *Enterobacteriaceae* were detected in all fecal samples, either in patients or healthy controls (Table 3).

Relative abundance of bacteria in IBD patients and healthy controls

Out of thirteen candidate bacterial families/genera that were investigated in this study, abundance of four families showed significant difference between the patients and control groups (Table 4; Fig. 1). No significant change was detected between UC and CD patients for amounts of these bacteria. Cut off value was calculated based on mean RQ of each bacterium as follows: *Bacteroides* (mean value: 1.80); *Faecalibacterium prausnitzii* (mean value: 1.76); *Prevotella* spp. (mean value: 433.6); *Methanobrevibacter* spp. (mean value: 0.56); Enterobacteriaceae (mean value: 3805); *Peptostreptococcus* spp. (mean value: 0.02); *Haemophilus* spp. (mean value: 50.20); and *Helicobacter pylori* (mean value: 7.92). It was showed that *Bacteroides* was significantly less abundant in IBD patients (0 to 18.01 fold) *vs* control group (0.5 to 21.67 fold, *p* value 0.0003). Similarly, *Faecalibacterium prausnitzii* (0 to 34.0165710 fold in patients' *vs* 0.0000303 to 20.1526432 in controls) and *Prevotella* spp. (0.0001148 to 9919.518555 fold in patients *vs* 0.1961566 to 29842.16602 fold in controls) showed lower abundance in IBD patients (*p* value 0.0001) (Fig. 2). While *Methanobrevibacterium* was more abundant in control group (279 fold *vs* 0.56 fold, *p* value 0.03), its frequency was low in both groups (6/29, 20.6% *vs* 1/29, 3%) (*p* value 0.03). No significant difference in abundance and frequency of members of *Enterobacteriaceae* and *Peptostreptococcus* spp. was detected in IBD patients compared with controls (0 to 63893.4023 fold *vs* 0 to 110217 fold in controls). However, increase in relative amounts of *Enterobacteriacea* (Mean RQ > 1 in compare to mean RQ < 1) was correlated with higher *IL8* levels in both the patients (3.6 fold increase in the population caused 1.1–7.6 fold increase in *IL8* level) and control groups (18 fold increase caused 1–2.8 fold increase in *IL8* level). Increase in relative amounts of *Haemophilus* (0 to 757.6660156 fold *vs* 0 to 275.7178650 fold), *Streptococcus* spp. (0.00064 to 34.219 fold increase), and *H. pylori* (0.0989889 to 168.1208038 fold *vs* 0 to 9.2172117 fold) were detected in IBD patients compared with controls, respectively; however these differences were not statistically significant. Statistical analysis was not performed for the other bacterial species, since low frequency of bacteria was detected among the patients and control groups. These bacteria were included *C. difficile* (0.1 fold in IBD patients *vs* 43.68 fold in controls), *Serratia* spp. (0.03 fold in a patient with UC), and *Klebsiella* spp. (21.35 fold in a patient with CD). Comparison of the obtained RQ levels showed lower abundances of *Bacteroides* (UC, *p* value = 0.02) and *Prevotella* spp. (CD) and higher amounts of *Haemophilus* (UC) and *Streptococcus* (UC [*p* value = 0.019], and CD) spp. in IBD patients who were with flares, in compare to those at remission stage. While these changes were detected in most of the

Table 4
Mean and standard deviation of relative abundance of bacteria in IBD patients' *vs* control group.

Bacteria	Case	Control	*p* value
Bacteroides	1.80 ± 4.5	3.25 ± 5.5	**0.0007**
Enterobacteriaceae	3805 ± 1327	4014 ± 2081	0.7
Faecalibacterium prausnitzii	1.76 ± 6.43	3.22 ± 5.26	**0.0002**
Haemophilus	50.20 ± 149.36	21.25 ± 54.11	0.41
Helicobacter pylori	7.92 ± 32.7	1.53 ± 2.07	0.35
Prevotella spp.	433.6 ± 1901.3	3059 ± 7392	**0.0001**
Methanobrevibacterium spp.	0.56 ± 3.008	279 ± 1398.7	**0.03**
Peptostreptococcus spp.	0.02 ± 0.1	0.15 ± 0.57	0.63

Table 3
Bacterial composition in IBD patients and healthy controls.

Co-colonization patterns	UC	CD	Control group
Common pattern: *Bacteroides* spp., *Prevotella* spp., *Streptococcus* spp., *Enterobacteriaceae, Helicobacter pylori, Haemophilus,* and *Faecalibacterium prausnitzii*	22 (100%)	7 (100%)	29 (100%)
Common pattern + *C. difficile*	1 (4%)	0	1 (3%)
Common pattern + *Methanobrevibacterium*	1 (4%)	0	6 (20%)
Common pattern + *Peptostreptococcus* spp.	2 (9%)	0	3 (10%)
Common pattern + *Peptostreptococcus* spp.+ *Serratia*	1 (4%)	0	0
Common pattern + *Klebsiella* spp.	0	1 (14%)	0

UC, ulcerative colitis; CD, Crohn's disease.

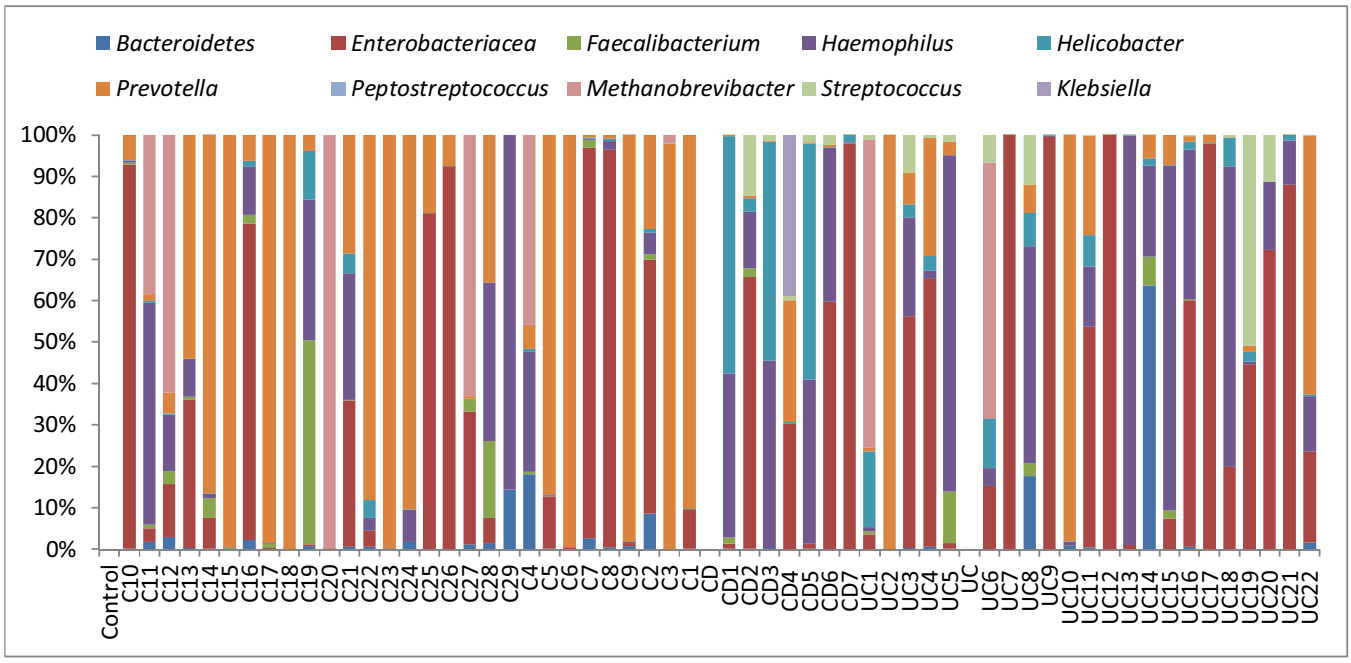


Fig. 1. Relative abundance of bacteria in fecal samples of IBD patients (UC, [UC1–22], and CD, [CD1-7]), and controls (C1-29).

patients, there were some variations between the patients at each phase. Lack of diversity in the bacterial composition of the patients with flares *vs* those at remission was also confirmed by Principal Coordinates Analysis (Fig. 3).

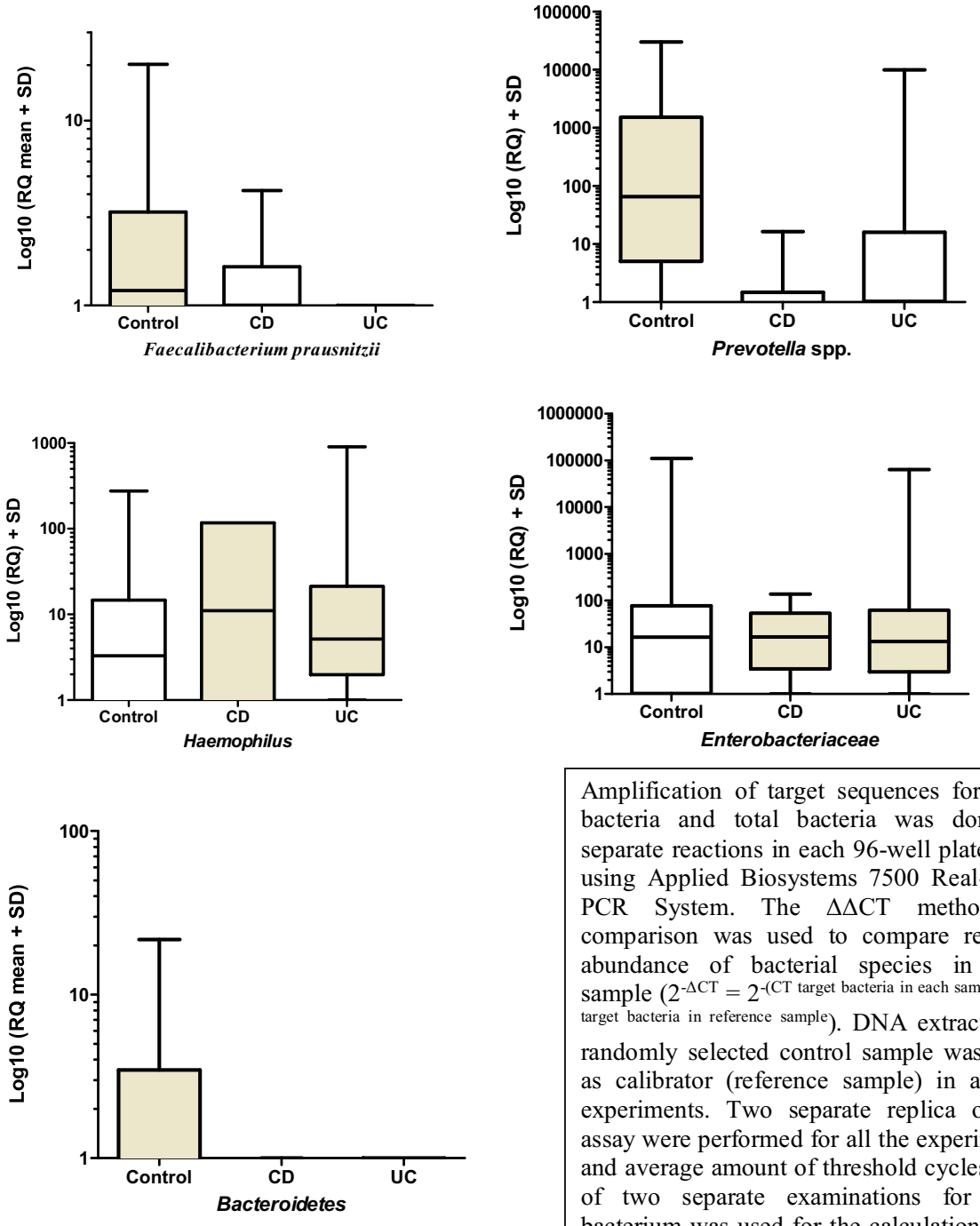
Increased level of IL8 *expression in the treated cell line with fecal microbiota of IBD patients compared with the controls*

Ability of the preserved filtrates of fecal samples from IBD patients and controls for induction of *IL8* expression was studied in differentiated HT-29 cells. Results of conventional RT-PCR for *ACTB* (Endogenous control) confirmed quality of the RNA extraction and cDNA synthesis procedures. As was shown in Fig. 4, expression of *IL8* was significantly increased in treated cells by inoculates of IBD patients compared with healthy controls (p value = 0.003). No significant difference in *IL8* mRNA levels was observed between the treated cells with fecal filtrates of the UC and CD patients.

Discussion

Association of infection with onset of IBD and its implication in the disease relapse was reported in previous studies. In one study, an increased incidence of infection with enteropathogens, especially *Clostridium* difficile, was shown in both UC and CD patients, which was approximately doubled and tripled in UC [40]. Intestinal epithelial cells are able to trigger innate immune responses against various microbes through immune receptor molecules, mainly pathogen recognition receptors (PRRs), which sense pathogen-associated molecular patterns (PAMPs) of the intestinal microbes. Inappropriate function of Toll-like receptors (TLRs) in IBD patients, as main PRRs of the intestinal epithelial cells, against members of intestinal microbiota could lead to activation of inflammatory pathways, induction of nuclear factor κB (NF-κB), release of pro-inflammatory and inflammatory cytokines (*e.g. IL8*), and other immune mediators that are involved in inflammatory reactions [41,42]. This inappropriate function could partly be in relation to the changes in the composition of gut microbiota and their immune stimulatory effects [43]. Our results showed significant difference in several classes of bacteria between the patients and control groups. This difference was correlated with disease activity of IBD and *in vitro IL8* transcription level in intestinal epithelial cells.

Persistent induction of the immune system as a result of dysbiosis could be supported by direct or indirect interaction of some bacterial species with mediators of the gut immune system, which thought to cause exacerbation of the disease in CD or UC patients. Ohkusa T. et al showed ability of some bacterial species isolated from ulcerative colitis (UC) patients, such as *Fusobacterium varium, Bacteroides vulgatus*, *Escherichia coli* and *Clostridium clostridioforme*, in induction of pro-inflammatory and inflammatory cytokines TNF-α, IL-6 and IL8 in SW-480 and HT-29 cell lines [44]. These cytokines are main mediators of inflammatory response and have crucial role in the pathogenesis of IBD [45]. Similarly, the increase and decrease in relative abundance of *Enterobacteriaceae*, *Pasteurellaceae* (*Haemophilus* spp.), *Veillonellaceae*, *Fusobacteriaceae*, and *Bacteroidales*, *Clostridiales*, and *Erysipelotrichales*, was reported in association to severity of Crohn's disease [46]. *Bacteroides fragilis* (*B. fragilis*) is the most common anaerobic organism in the stool samples. In a study by Mazmanian SK. et al., it was reported that *B. fragilis*protects animals from experimental colitis. This beneficial activity exerts by polysaccharide A (PSA), whichprotects from inflammatory disease through induction of an anti-inflammatory cytokine [47,48]. In congruence with these findings, our results showed that the mean value of *Bacteroides* spp. was significantly decreased in both UC and CD patients compared with healthy controls. In our study, decrease in amount of *Faecalibacterium* spp., the other member of the human gut microbiota, was also measured in IBD patients. Similarly, in a study by Sokol et al. a lower abundance of *F. prausnitzii* in the ileal mucosa was reported in patients with recurrent Crohn's disease. Anti-inflammatory effect of *F. prausnitzii* through inhibition of NF-κB activation and *IL8* production was established in both *in vitro* and *in vivo* models [49,50]. This anti-inflammatory effect could also induced by alteration of the other members of the gut microbiota. Our findings showed decreased amount of *Prevotella* and *Methanobrevibacter* spp. in fecal microbiota of the patients. Anti-inflammatory effect of *P.* histicola through suppression of



Amplification of target sequences for each bacteria and total bacteria was done in separate reactions in each 96-well plate read using Applied Biosystems 7500 Real-Time PCR System. The ΔΔCT method of comparison was used to compare relative abundance of bacterial species in each sample ($2^{-\Delta CT} = 2^{-(CT\ target\ bacteria\ in\ each\ sample\ -\ CT\ target\ bacteria\ in\ reference\ sample)}$). DNA extract of a randomly selected control sample was used as calibrator (reference sample) in all the experiments. Two separate replica of the assay were performed for all the experiments and average amount of threshold cycles (C_T) of two separate examinations for each bacterium was used for the calculation. This amount was presented as fold change for all the bacteria.

Fig. 2. Altered population of bacteria with greatest difference between stool samples of the IBD patients compared with healthy controls.
Legend of Fig. 2. Amplification of target sequences for each bacteria and total bacteria was done in separate reactions in each 96-well plate read using Applied Biosystems 7500 Real-Time PCR System. The ΔΔCT method of comparison was used to compare relative abundance of bacterial species in each sample ($2^{-\Delta CT} = 2^{-(CT\ target\ bacteria\ in\ each\ sample\ -\ CT\ target\ bacteria\ in\ reference\ sample)}$). DNA extract of a randomly selected control sample was used as calibrator (reference sample) in all the experiments. Two separate replica of the assay were performed for all the experiments and average amount of threshold cycles (C_T) of two separate examinations for each bacterium was used for the calculation. This amount was presented as fold change for all the bacteria.

inflammatory cytokine was shown in the jejunum and colon of P. histicola–treated mice. This effect was associated with generation of Treg cells and increased expression of IL-10, which could caused to lower expression of IL8 in the intestine [51]. There are contrary reports about abundance of *Methanobrevibacter* spp. in IBD patients. While increased prevalence of *M.* stadtmanae in these patients was reported by Blais-Lecours P. et al. [52] that was correlated with their ability for induction of inflammatory cytokines [53], Pascal V. et al. and Ghavami SB. et al. presented a decrease in the population of this species among both UC and CD

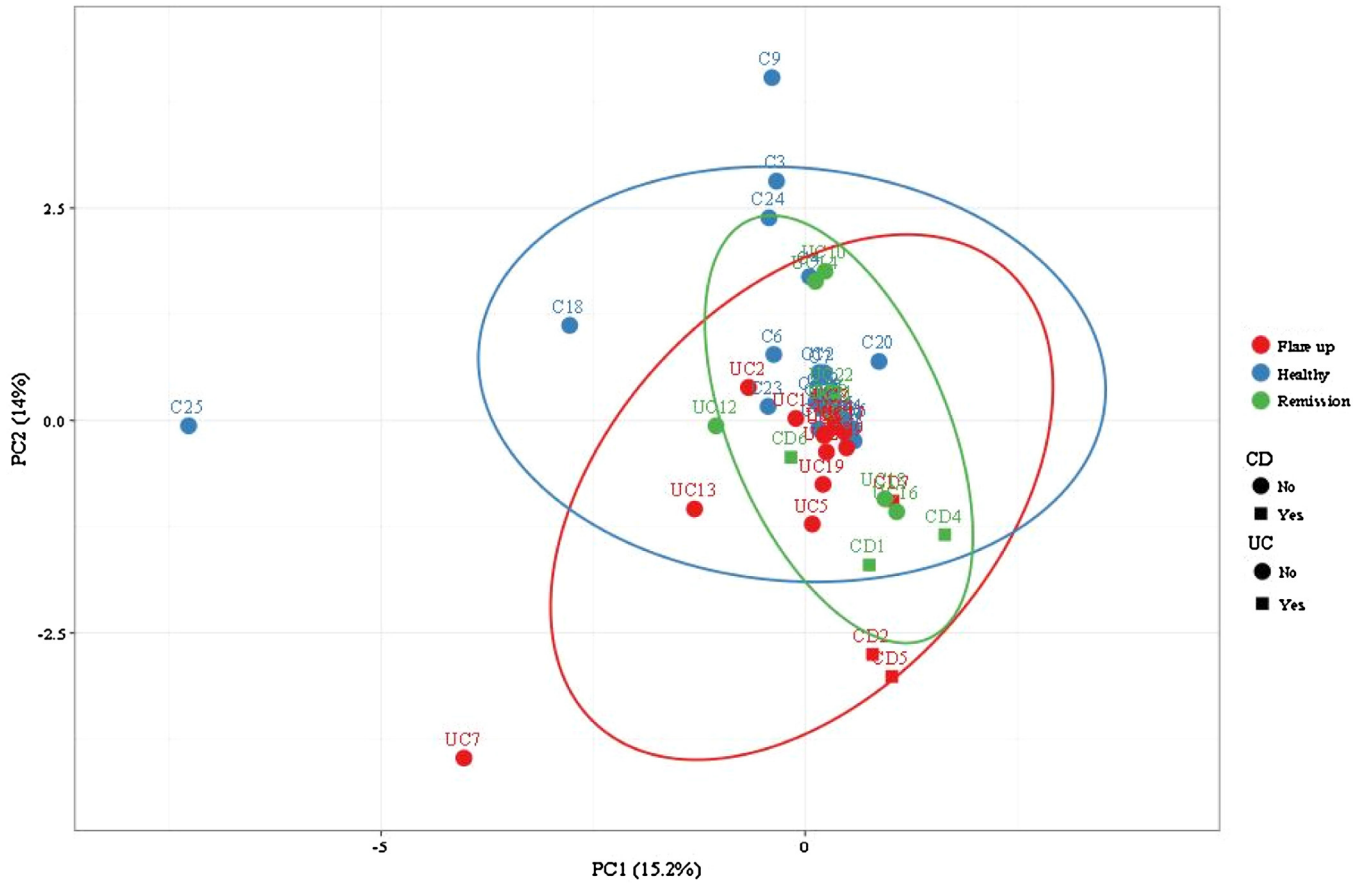


Fig. 3. Bacterial community clustering using Principal Coordinates Analysis (PCoA) in IBD (UC and CD) patients and controls.
Legend of Fig. 3: Unit variance scaling was applied to rows; SVD with imputation was used to calculate principal components. X and Y axis show principal component 1 and principal component 2 that explain 15.2% and 14% of the total variance, respectively. Prediction ellipses are such that with probability 0.95, a new observation from the same group will fall inside the ellipse. Each point corresponds to a community colored according to disease location.

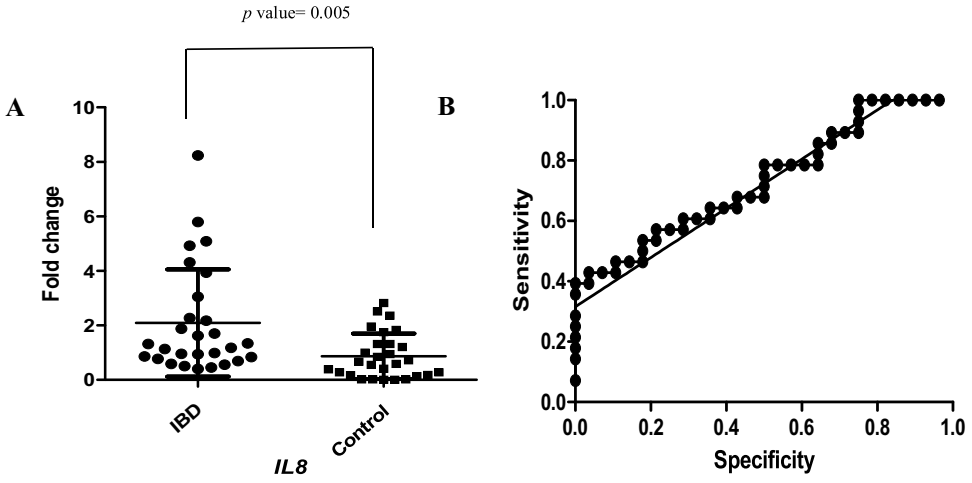


Fig. 4. *IL*-8 expression in IBD patients *versus* healthy controls. A. Dot diagram of *IL*-8 expression in IBD patients *versus* healthy controls; B. Receiver operating characteristic (ROC) curve analysis.
Legend of Fig. 4: Relative abundance of the bacteria in the control and patient groups and changes of *IL8* gene expression at mRNA levels was analyzed by ABI 7500 software. Statistical difference between these groups was determined by GraphPad prism 5 using unpaired t-test. Receiver-Operator Characteristic (ROC) curve was drawn to show sensitivity and specificity of the test for differentiation of IBD patients from control people. In the case of *IL8*, RQ values of less than 0.5 fold change were considered as decreased expression and those higher than 2 fold increase were defined as increased expression.

patients [54,55]. Results of our study showed significant decrease in relative abundance of this member of Archaebacteria in our patients. Comparison of IL8 levels in the control and patients groups proposed an anti-inflammatory role for this bacterium, since lower expression level was detected in the cases with lower counts of Methanobrevibacterium. The correlation between IL8 level and relative abundance of the other bacterial species was not confirmed in both the control and IBD samples.

Due to the existence of the mucosal immunity in the gastrointestinal tract, interaction of *H. pylori* with the immune system could arise systemic adverse effects in distal organs. There are increasing data supporting the existence of a correlation between *Helicobacter pylori* infection and extragastric disorders [56]. This association in the case of IBD is controversial. In a study by Luther J. et al., they revealed inverse association between H. pylori colonization and IBD [57]. Contrary to their results, our study showed that relative abundance of *H. pylori* was increased in IBD patients compared with healthy controls (>8 fold increase), but this increase was not statistically significant. Since *H. pylori* infection has high prevalence in general population of Iran (30->80%) [58], the higher abundance of this bacterium in the patients group compared with the controls proposed a causal role for this bacterium in the pathogenesis of IBD.

Seth M.B. et al. demonstrated that intestinal inflammation is correlated with overgrowth of *Enterobacteriaceae*, but it is unclear yet whether abundance of *Enterobacteriaceae* is a cause or effect of the disease [59]. In current study we similarly showed that *Enterobacteriaceae* was increased in IBD patients compared with healthy controls, but this increase was not statistically significant. Higher abundance of *Enterobacteriacea* in stool samples of studied samples was correlated with higher level of *IL8* transcription in the treated cells, which was prominent in the patients' compared with the control group.

Increased amount of *Peptostreptococcus* spp. in the intestine of CD patients was reported by Verma R. et al., but not in UC patients [25]. However, in our study we showed that there is no significant difference between the abundance of *Peptostreptococcus* spp. in UC patients and healthy controls. While correlation between asymptomatic carriage of *Campylobacter* spp. and occurrence of IBD is not so clear, Kim O. et al. showed that the risk of IBD could increase after the onset of infection with this bacterial species [60]. Results of our study didn't confirm such correlation in our patients.

Relationship between the reduction of bacterial families and BMI was shown for *Haemophilus* in the IBD patients. Accordingly, lower amounts of these bacteria were detected in patients with higher BMI. Inverse association between *Haemophilus* and body fat percentage was previously shown by Bressa C. et al. [61]. Similarly, a decrease in *Haemophilus* spp. was shown to be linked with the development of type II diabetes [62], and rheumatoid arthritis, an inflammatory disease [63]. In the case of other bacteria we didn't find such correlation. Reduction in the *Bacteroides* population was more frequent in underweight patients in CD group compared with UC group.

A relationship between disease activity in UC patients and abundance of bacterial families was found in our study. Accordingly, the decreased relative amounts of bacterial families, including *Bacteroides* spp., *Faecalibacterium prausnitzii*, and *Prevotella* spp., were found in UC patients with disease activity score greater than 4. However, this finding was not supported by the level of *IL8* expression detected in the stool samples of these patients. The inverse correlation between decreased level of *Bacteroides* in the gut microbiota and active UC and CD phase was similarly confirmed by Zhou Y. et al [64]. Association of disease phase and relative amounts of the studied bacteria showed higher levels of *Streptococcus* and *Haemophilus* and decreased amounts of *Bacteroides* and *Prevotella* spp. in patients who were at flares *vs* those at remission phase. There are only few studies that compared microbial composition changes during remission and flare up of IBD. Contrary to our results, overall increase in *Bacteroidetes* and *Prevotella* spp., in patients with flares was reported by a recent study [65]. However, variations of results between the patients was reported in another study during the remission and active phases [66].

While results of this study improved our understanding about correlation of defined members of the intestinal bacteria with IBD, main limitations of this cross-sectional investigation may summarized as inclusion of patients with ulcerative colitis and Crohn's disease in a single study, doing the study on defined members of bacterial families instead of measuring complete pattern of the microbiome using next generation sequencing techniques, the small number of patients, and existence of diversity in the prescribed treatment regimens and the disease stages among them. Additional studies will be needed to determine correlation of the changes detected in the intestinal microbiota and their functional effects on the occurrence or exacerbation of the disease. In conclusion, although larger patient numbers need to be studied, these findings showed changes of fecal microbial composition in IBD patients compared with healthy people. This difference was also the case for IBD patients who were in flares compared with those in remission. These results showed that changes in the fecal microbiota composition could affect disease activity, which is correlated with increased level of *IL8* induction.

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