



Association of altered gut microbiota composition with chronic urticaria

Edris Nabizadeh, MSc^{*}; Nima Hosseini Jazani, PhD^{*}; Morteza Bagheri, PhD[†]; Shahram Shahabi, MD, PhD[†]

^{*} Faculty of Medicine, Department of Microbiology, Urmia University of Medical Sciences, Urmia, Iran

[†] Cellular and Molecular Research Center, Urmia University of Medical Sciences, Urmia, Iran

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ABSTRACT

Background: An altered gut microbiota composition has recently been linked to some types of allergies.

Objective: To compare the relative amounts of *Akkermansia muciniphila*, *Clostridium leptum*, *Faecalibacterium prausnitzii*, and Enterobacteriaceae as members of gut microbiota among patients with chronic urticaria (CU) and healthy controls.

Methods: A total of 20 patients with CU and 20 healthy individuals matched by age and sex participated in the study. Fresh fecal samples were collected, and DNA extracted from stool samples was analyzed by real-time polymerase chain reaction for the qualitative and quantitative assays of the so-called bacteria.

Results: The frequencies of *A muciniphila*, *C leptum*, and *F prausnitzii* in healthy controls' stool samples were significantly more than those of patients with CU ($P < .001$, $P < .01$, and $P < .05$, respectively), whereas the Enterobacteriaceae family was detected in all patients and healthy controls' stool samples. The relative amounts of *A muciniphila* in healthy control positive samples were significantly higher than those of samples from patients with CU ($P < .001$). Furthermore, there was a corresponding increase of relative amounts of *C leptum* and *F prausnitzii* in healthy control positive samples compared with those of patients with CU ($P = .09$ and $P = .08$, respectively). The mean of the relative amounts of Enterobacteriaceae family in the stool samples from patients with CU was more than that of healthy controls; however, the difference was nearly significant ($P = .12$).

Conclusion: The results reveal a change of frequency and relative amounts of *A muciniphila*, *C leptum*, and *F prausnitzii* in patients with CU compared with healthy controls. This is the first study, to our knowledge, to show the change of microbiota composition in patients with CU.

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Introduction

Chronic urticaria (CU) is a relatively common disease that can significantly affect the quality of life.¹ CU is defined as urticaria that is continuously or intermittently present for at least 6 weeks.² The precise cause of CU is largely unknown, and the major therapy is symptomatic control.³ CU is a systemic immune-mediated disease,⁴ so it is highly possible to expect an imbalance in immune system, leading to CU in forms with and without a known cause.

Gut microbiota plays an important role in evolution and regulation of the immune system.⁵ The implication of microbiota in

many immune-mediated diseases has been reported,⁶ for example, diabetes,⁷ Crohn disease,⁸ ulcerative colitis,⁸ multiple sclerosis,⁹ uveitis,¹⁰ various cancers,¹¹ asthma,¹² food allergy,^{13,14} coronary artery disease,¹⁵ and atopic eczema.¹⁴

Akkermansia muciniphila is a strictly anaerobic, gram-negative, oval-shaped bacterium that belongs to the phylum of Verrucomicrobia, which is highly active in the degradation of mucin.^{16,17} *A muciniphila* is present in high numbers in the gastrointestinal tract of human and several mammalian species, especially in the cecum, where most amounts of mucin are produced.¹⁸ The anti-inflammatory effects of *A muciniphila* in the prevention and control of some diseases in humans, including obesity, diabetes type 2,^{19–22} appendicitis,²³ irritable bowel syndrome,²⁴ autism,²⁵ and atherosclerosis,²⁶ have been well established.

Faecalibacterium prausnitzii, one of the most common gastrointestinal bacteria, is a gram-positive bacterium that belongs to *Clostridium leptum* group from the Firmicutes phylum. Several

Reprints: Shahram Shahabi, MD, PhD, Cellular and Molecular Research Center, Urmia University of Medical Sciences, Urmia, Iran; E-mail: s_shahabi@umsu.ac.ir.

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studies have found lower amounts of this bacterium in some diseases, such as allergy,²⁷ atopic dermatitis,²⁸ chronic kidney disease,²⁹ inflammatory bowel disease,³⁰ major depression,³¹ obesity,³² and Crohn disease.^{33,34}

The class of Clostridia includes the anaerobic, gram-positive, endospore-forming bacteria belonging to the Firmicutes phylum. Although some Clostridia groups include pathogenic species, most of them are commensal residents of human and some animals' gastrointestinal tracts.³⁵ Cluster IV of Clostridia (also known as *C leptum*) is composed of 4 genera, including *Clostridium*, *Eubacterium*, *Ruminococcus*, and *Anaerofilum*.³⁶ This cluster is also one of the most predominant organisms in the colon.³⁷ The protective role of these bacteria against atopic diseases and inflammatory states has been demonstrated.^{38–41}

The Enterobacteriaceae is a large family of gram-negative bacilli, which includes many harmless and several pathogenic genera. Members of the Enterobacteriaceae are important parts of gut microbiota, so they can be referred to as enteric bacteria.⁴² An increase in the population of Enterobacteriaceae has been reported in gut microbiota in several inflammatory diseases, including food sensitization,⁴² atopic allergy,³⁸ bowel inflammatory disease, and celiac disease,⁴³ indicating the role of this bacterial family in induction of immune deviation.

CU is an immune-mediated disease, so an association between the composition of gut microbiota and CU is possible. The present study aimed to compare the presence and the relative amounts of some members of gut microbiota among patients with CU vs healthy controls. The examined bacteria included *C leptum*, *A muciniphila*, *F prausnitzii*, and Enterobacteriaceae. To the best of our knowledge, this is the first study on the implication of microbiota in CU.

Methods

Study Design and Participants

Forty people (20 patients with CU and 20 healthy controls) were enrolled in the study. Every patient had an age- and sex-matched healthy control. CU was diagnosed and assessed according to the American Academy of Allergy, Asthma, and Immunology criteria.² Patients and healthy controls should not have used any antibiotics at least 1 month before entering the study. Other exclusion criteria were C1 esterase inhibitor deficiency, lymphocytopenia, thrombocytopenia, and any other known disease for the patients and any known disease for the healthy controls. Demographic information was collected from all participants. The study was performed with the approval of research ethics committee of Urmia University of Medical Sciences, Urmia, Iran, and all the patients and healthy individuals signed written informed consent forms. All the study procedures and the participants were in accordance with the Declaration of Helsinki (1964) and its later amendments.

Sample Collection and DNA Extraction

Approximately 2 g of fresh stool samples obtained during a 9-month period from May 1, 2015, through December 31, 2015, were collected in a sterile plastic cup and kept in an icebox using a standard collection method. DNA was extracted from 200 ± 20 mg of feces using a QIAamp DNA stool mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The purity of extracted genomic DNA was confirmed via spectrophotometric analyses (A260/280 ratio of 1.8). All extracted DNA samples were stored at –20°C until further analysis.

Polymerase Chain Reaction

The bacterial genomic DNA was amplified with specific primers for *A muciniphila*,⁴⁴ *C leptum* (cluster IV),⁴⁵ Enterobacteriaceae,⁴⁶ and *F prausnitzii*⁴⁷ and universal bacteria primer 16s ribosomal

RNA gene as housekeeping gene,⁴⁸ as described elsewhere. Polymerase chain reaction (PCR) was performed in a reaction volume of 25 µL for each sample, containing genomic DNA (50 ng), PCR Master Mix (12.5 µL) with PCR buffer, recombinant Taq polymerase, magnesium chloride and deoxynucleotide (CinnaGen, Tehran, Iran), deionized distilled water (9.5 µL), and 10 pM of each primer (1 µL). Cycling conditions included 1 cycle at 95°C for 10 minutes and 40 cycles at 94°C for 20 seconds, 60°C for 60 seconds, and 72°C for 60 seconds. Negative controls were included in each PCR to avoid contamination. PCR-amplified products were detected by electrophoresis on 1.5% agarose gel. A 100–base pair (bp) DNA Ladder (SinaClon, Tehran, Iran) was used for determining the molecular weight of produced bands.

Quantitative Real-Time PCR

The bacterial relative amounts in positive stool samples were determined by quantitative real-time PCR (qPCR), using the same primers of PCR reactions and specific probes (Table 1). The qPCR assays were performed in 96-well optical plates with Real Q Plus 2× Master Mix for Taq Man Probe (Viragene, Tehran, Iran) on an IQ5 multicolor Real-time PCR Detection System (Bio-Rad Laboratories, Hercules, California): 0.5 µL (5 pM concentration) of each primer, 0.5 µL (5 pM concentration) of each probe, and 1 µL (50 ng) of template DNA in a final volume of 25 µL per reaction. Cycling conditions were 96°C for 10 minutes for initial denaturation and Taq polymerase activation and 40 cycles at 95°C for 30 seconds and 60°C for 1 minute. DNA-free water was used as a negative control. The comparative C_T method was used to determine bacterial relative amount in each sample through calculating 2^{–ΔΔC_T}.

Statistical Analysis

Frequencies and relative amounts of the bacteria were compared between two groups using the Mann-Whitney test and independent sample *t* test, respectively.

Results

There were no significant differences between the 20 patients with CU and the 20 healthy controls with respect to mean (SD) age (37.7 [3.46] years in patients with CU and 37.55 [3.55] years in healthy controls; *P* > .99) and sex (8 men and 12 women in each group; *P* = .98). Initial PCR was performed for all DNA samples extracted from the feces of patients with CU and healthy controls to

Table 1

Primer Sets Used for PCR and RT-qPCR of *Akkermansia muciniphila*, *Clostridium leptum*, *Faecalibacterium prausnitzii*, and Enterobacteriaceae

Amplified gene target, bp	Primers	Bacterial group
329	Forward: CAGCACGTGAAGGTGGGGAC Reverse: CCTTGGCGTTGGCTTCAGAT Probe: CCTTGGCGTTGGCTTCAGAT	<i>Akkermansia muciniphila</i>
115	Forward: CCTTCCGTGCCGAGTTA Reverse: GAATTAACACATACTCACTGCTT Probe: CACAATAAGTAATCCACC	<i>Clostridium leptum</i> (cluster IV)
69	Forward: GCGGTAGCACAGAGCTT Reverse: GGCAGTTTCCAGACATTACTCA Probe: CCGCGCTCGTCACC	Enterobacteriaceae
158	Forward: TGGAGCATGTGGTTAATTCGA Reverse: TGGCGGACTTAACCCAACA Probe: CACGAGCTGACGACA[A/G]CCATGCA	Universal
198	Forward: GATGGCCTCGCGTCCGATTAG Reverse: CCGAAGACCTTCTCTCTCC Probe: CACCAGTTTACCTTCGG	<i>Faecalibacterium prausnitzii</i>

Abbreviation: bp, base pairs.

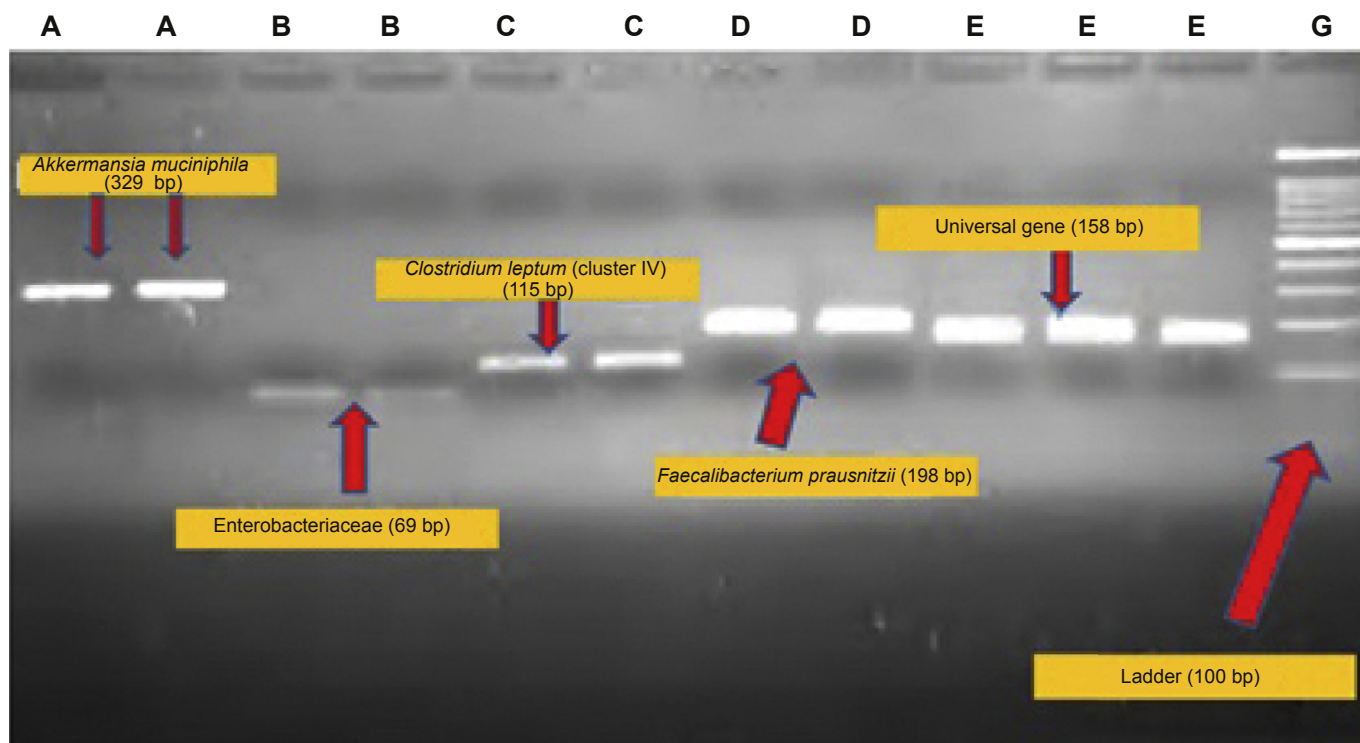


Figure 1. Electrophoresis of polymerase chain reaction products of the studied bacterial specific gene amplification on agarose gel 1.5%. A, *Akkermansia muciniphila* (329 base pairs [bp]); B, Enterobacteriaceae (69 bp); C, *Clostridium leptum* (cluster IV) (115 bp); D, *Faecalibacterium prausnitzii* (198 bp); E, universal gene (158 bp); G, GeneRuler DNA Ladder (100 bp) (Thermo Fisher Scientific, Waltham, Massachusetts).

investigate the presence of *A. muciniphila*, Enterobacteriaceae, *C. leptum* (cluster IV), and *F. prausnitzii* (Fig 1).

The results indicated that the frequencies of *A. muciniphila*, *C. leptum*, and *F. prausnitzii* in healthy controls' stool samples were significantly more than those of patients with CU ($P < .001$, $P < .01$, and $P < .05$, respectively), whereas the Enterobacteriaceae family

were detected in all patients and healthy controls' stool samples (Fig 2).

Figure 3 shows that the mean of the relative amounts of *A. muciniphila* in the positive samples of healthy controls were significantly higher than those in the samples of patients with CU ($P < .001$). There was a nearly significant ($P = .09$) increase in the

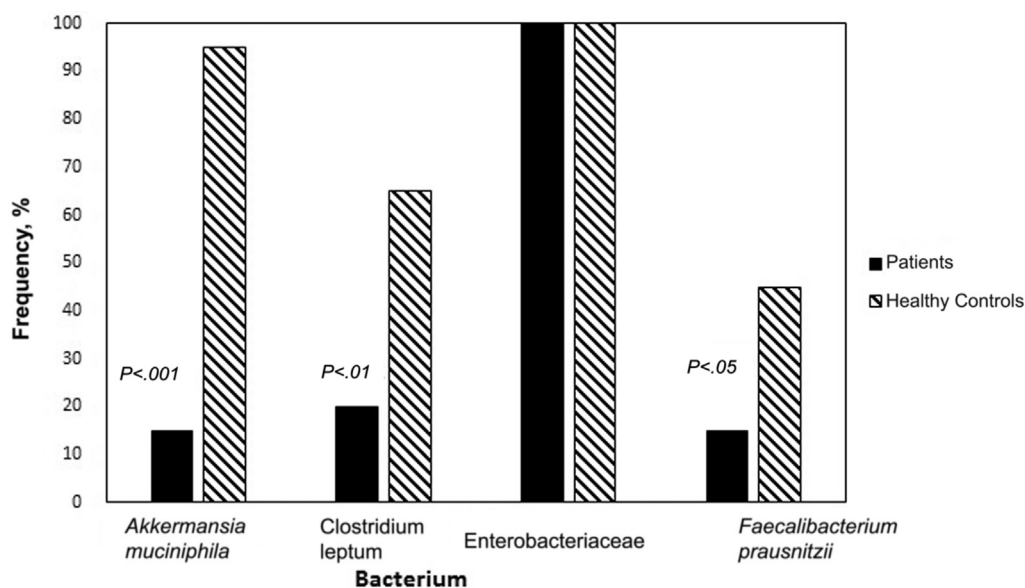


Figure 2. Frequencies of the studied bacteria in the stool samples of patients with chronic urticaria (CU) and healthy controls; initial polymerase chain reaction was performed for all DNA samples extracted from the feces of patients with CU and healthy controls to investigate the presence of *Akkermansia muciniphila*, Enterobacteriaceae, *Clostridium leptum* (cluster IV), and *Faecalibacterium prausnitzii*. The frequencies of *A. muciniphila*, *C. leptum*, and *F. prausnitzii* in the stool samples of healthy controls were significantly more than those of patients with CU ($P < .001$, $P < .01$, and $P < .05$, respectively), whereas the Enterobacteriaceae family were detected in the stool samples of all patients and healthy controls.

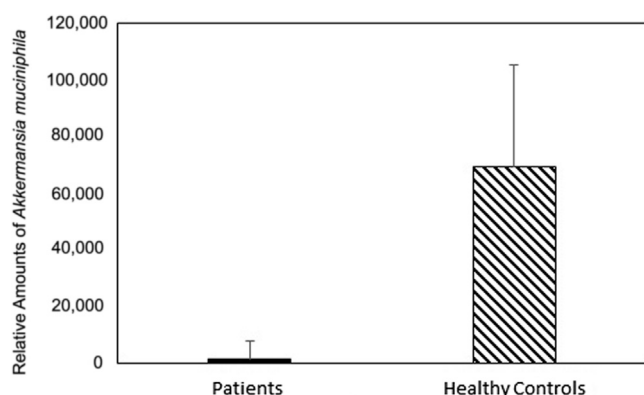


Figure 3. The relative amounts of *Akkermansia muciniphila* in the fecal microbiota of patients with chronic urticaria (CU) and healthy controls; the bacterial relative amounts were determined by quantitative polymerase chain reaction in positive stool samples by the method explained in the text. The mean relative amount of *A muciniphila* in the positive samples from healthy controls was significantly higher than that of patients with CU ($P < .001$).

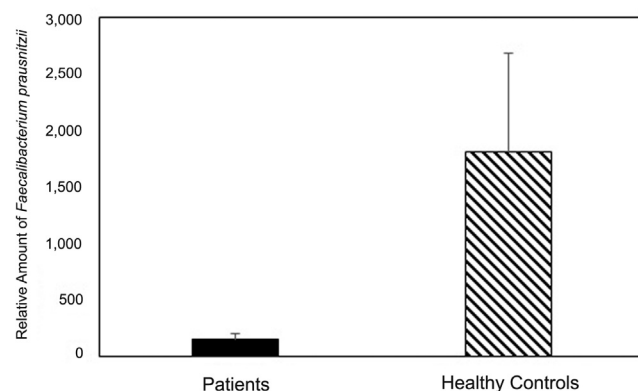


Figure 5. The relative amounts of *Faecalibacterium prausnitzii* in the fecal microbiota of patients with chronic urticaria (CU) and healthy controls; the bacterial relative amounts were determined by quantitative polymerase chain reaction in positive stool samples by the method explained in the text. There was an increase ($P = .08$) in the mean relative amount of *F prausnitzii* in the positive samples of health controls compared with patients with CU.

relative amounts of *C leptum* in the positive samples of healthy controls compared with patients with CU (Fig 4). The mean relative amount of *F prausnitzii* in the positive samples from healthy controls was higher than that of the samples from patients with CU, but the difference was nearly significant ($P = .08$) (Fig 5). The mean relative amount of the Enterobacteriaceae family in the stool samples from the patients with CU was more than that of healthy controls, but the difference was almost significant ($P = .12$) (Fig 6).

Discussion

The results of this study indicate that the frequencies of *A muciniphila*, *C leptum*, and *F prausnitzii* in healthy controls' stool samples were significantly more than in patients with CU, whereas the Enterobacteriaceae family was detected in the stool samples of all patients and healthy controls. The relative amounts of *A muciniphila* in healthy control positive samples were higher than those of patients with CU. Furthermore, there was a corresponding increase of relative amounts of *C leptum* and *F prausnitzii* in positive samples from healthy controls compared with those of patients with CU. However, the relative amounts of the Enterobacteriaceae

family in the stool samples from patients with CU were more than those of healthy controls.

The frequency and relative amounts of *A muciniphila*, *C leptum*, and *F prausnitzii* in healthy controls were significantly more than those of patients with CU, which is in line with the findings of previous studies. For example, Candela et al³⁸ found a significant depletion in members of *A muciniphila*, *C leptum*, and *F prausnitzii* in children with a clinical diagnosis of allergy (asthma, rhinitis, grass pollen sensitization, oral allergy syndrome, allergic atopic dermatitis, and cow's milk allergy). Other studies found a reverse correlation between these bacteria and inflammatory states.^{16,39,40,44,49} Because CU is an inflammatory state, a reverse correlation found between these bacteria and CU is in line with the so-called studies. Some mechanisms can be suggested for the protective effects of these bacteria against CU, including induction of regulatory T (Treg) cells. This mechanism is supported by other studies that revealed that these bacteria could induce Treg cells.^{50–54} Treg cells can reduce inflammation through secretion of anti-inflammatory mediators,⁵⁵ and an anti-inflammatory milieu is protective against CU. This mechanism is in line with studies that found reduced

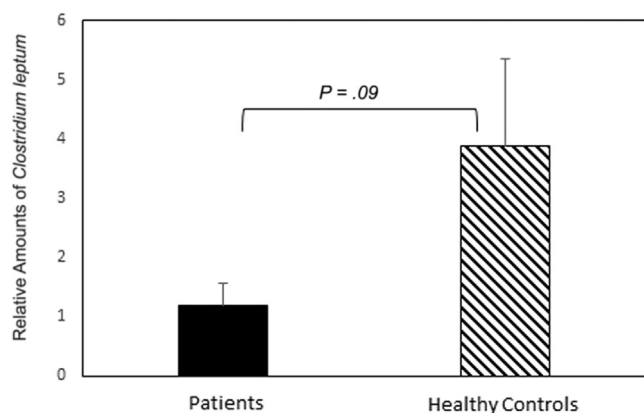


Figure 4. The relative amounts of *Clostridium leptum* in the fecal microbiota of patients with chronic urticaria (CU) and healthy controls; the bacterial relative amounts were determined by quantitative polymerase chain reaction in positive stool samples by the method explained in the text. There was an increase ($P = .09$) in the mean of the relative amount of *C leptum* in the positive samples of healthy controls compared with that of patients with CU.

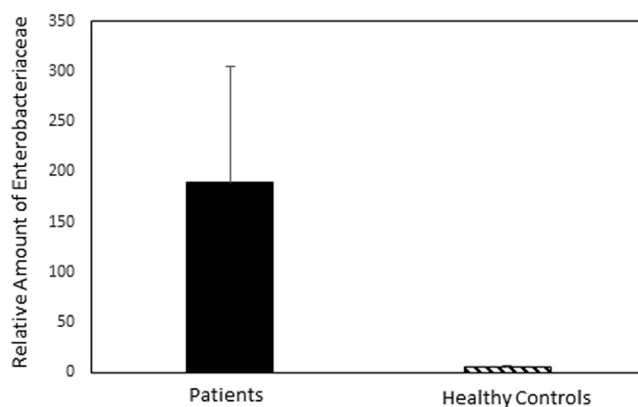


Figure 6. The relative amounts of the Enterobacteriaceae family in the fecal microbiota of patients with chronic urticaria (CU) and healthy controls; the bacterial relative amounts were determined by quantitative polymerase chain reaction in positive stool samples by the method explained in the text. The mean relative amount of the Enterobacteriaceae family in the stool samples of patients with CU was more than that of healthy controls but the difference was nearly significant ($P = .12$).

numbers⁵⁶ and function⁵⁷ of Treg cells in patients with CU. Some other mechanisms, such as improvement of gut barrier function^{58,59} and increased production of butyrate,^{35,60} may be considered as well.

The current findings indicated no difference between the frequency of the Enterobacteriaceae family in the patients with CU and the healthy controls, but the relative amounts of these bacteria were larger in the patients with CU compared with the healthy controls. This finding is in agreement with studies that reported a nonprotective or antiprotective role for the Enterobacteriaceae family against eczema, inflammatory bowel disease, food allergy, and atopy.^{42,61–63} This finding can be explained by the fact that the Enterobacteriaceae family members are among proinflammatory members of gut microbiota.³⁸

Decrease in the frequency and the relative amounts of *A muciniphila*, *C leptum*, and *F prausnitzii* and increase of the relative amounts of the Enterobacteriaceae family in patients with CU compared with healthy controls were observed. This is the first study, to our knowledge, that found change of microbiota in patients with CU; however, subsequent studies are required to confirm the findings.

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