Original Article

Reduced abundance of butyric acid-producing bacteria in the ileal mucosa-associated microbiota of ulcerative colitis patients

Motoyasu Osawa,[1] Osamu Handa,[1,*] Shinya Fukushima,[1] Hiroshi Matsumoto,[1] Eiji Umegaki,[1] Ryo Inoue,[2] Yuji Naito,[3] and Akiko Shiotani[1]

[1]Department of Internal Medicine, Division of Gastroenterology, Kawasaki Medical School, 577 Matsushima, Kurashiki, Okayama 701-0192, Japan
[2]Faculty of Agriculture, Setsunan University, 45-1 Nagaotoge-cho, Hirakata, Osaka 573-0101, Japan
[3]Department of Human Immunology and Nutrition Science, Kyoto Prefectural University of Medicine, 465 Kajii-cho, Kawaramachi Hirokoji Agaru, Kamigyo-ku, Kyoto 602-8566, Japan

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Compositional changes in the microbiota are associated with various inflammatory diseases, including ulcerative colitis (UC). Aim: This study aimed to investigate the mucosa-associated microbiota (MAM) in patients with UC and its difference related with disease activity and classification. Brush samples were collected from the terminal ileum and sigmoid colon during endoscopic procedures. The microbiota of samples was profiled using the Illumina MiSeq platform. The V3–V4 regions of the gene encoding 16S rRNA (460 bp) were amplified using PCR. Fifty UC patients and twenty healthy controls were enrolled. UC patients displayed significantly reduced α-diversity in both the ileum and sigmoid colon compared to controls. A difference in β-diversity in the unweighted analysis was observed between the two groups. The abundance of *Lactobacillus* and *Veillonella* was significantly higher and that of *Butyricicoccus*, *Ruminococcus* and *Lachnospiraceae* was significantly lower in the ileum of UC patients than in controls. The abundance of *Odoribacter* in the ileum was significantly lower in left-sided colitis and pancolitis patients than in proctitis patients and lower in patients with highly severe disease activity than with mild disease activity. The reduction in abundance of butyric acid-producing bacteria, especially *Odoribacter*, in ileal MAM may play an important role in the pathophysiology of UC.

Key Words*:** **ulcerative colitis, mucosa-associated microbiota, butyric acid-producing bacteria, lactic acid-producing bacteria, *Odoribacter

Ulcerative colitis (UC) is an inflammatory bowel disease (IBD) that causes recurrent gastrointestinal mucosal inflammation and reduces the quality of life of patients. The commensal microbiome plays an important role in nutrient metabolism, protection from pathogens, immune system development, and the pathogenesis of various diseases,[1] including UC. Dysbiosis of the gut microbiome, a lower abundance of obligate anaerobic bacteria, a decrease in the levels of metabolites and short-chain fatty acids (SCFAs), and an increase in the abundance of facultative anaerobes are also associated with IBD.[2,3] Emerging evidence indicates that gut microbes are an important factor in modulating the immune responses that affect IBD progression. Therefore, therapeutic microbial manipulation with fecal microbiota transplantation (FMT) is considered effective in the treatment of patients with UC by increasing microbial diversity and the production of SCFAs.[4]

A high concentration of mucosal bacteria in UC patients has previously been observed using biopsy samples.[5] The reduced bacterial diversity and Firmicutes abundance and the increased levels of Gammaproteobacteria have also been reported in UC patients.[6] Intraluminal microorganisms and mucosa-associated microbiota (MAM) are the two groups of organisms that make up the gut microbiota. MAM are bacterial microbiota that adhere to the intestinal mucosa and mucus. [7] These bacteria are thought to affect epithelial and mucosal function and may be more deeply involved in the pathophysiology of IBD.[8] In our previous human study,[9] the bacterial profiles of stool samples were found to be significantly different from those of MAM obtained using brush forceps during endoscopic procedures. Moreover, butyric acid-producing bacteria, including *Coprococcus*, *Roseburia*, and *Ruminococcus*, were significantly less abundant in patients with Crohn's disease (CD) than in healthy individuals.[10] Butyric acid confers anti-inflammatory and immune regulatory effects through the production of interleukin-10 (IL-10) from dendritic cells and induction of Foxp3+T regulatory cells (Tregs).[11]

The purpose of this study was to compare MAM profiles of the ileum and sigmoid colon between Japanese UC patients and healthy control individuals. The difference in the MAM profiles related with disease activity and classification was also investigated. Our findings provide insights into the association of butyric acid-producing bacteria with pathophysiology of UC.

Material and Methods

Patients and sample collection. The enrolled subjects were as follows: healthy controls undergoing routine medical checkups and UC patients. As healthy controls, we excluded those who received any medication or complained of any gastrointestinal symptoms and patients with tumor lesions or inflammatory lesions found by colonoscopy. UC was classified into three different types (proctitis, left-sided colitis, and pancolitis) based on the location of inflammation within the colon. Disease severity was evaluated based on the Mayo endoscopic sub-score (MES) consisting of a 4-point scoring system; scores ranged from 0 to 3 [0 = normal or inactive disease; 1 = mild disease (erythema, decreased vascular pattern); 2 = moderate disease (marked erythema, absent vascular pattern, friability, erosions); 3 = severe disease (spontaneous bleeding, ulceration)].

Brush samples were taken from less inflamed mucosa in the terminal ileum (30 cm from Bauchin's valve) and the sigmoid colon using an endoscopic microbiology brush (COOK,

*To whom correspondence should be addressed.
E-mail: handao@med.kawasaki-m.ac.jp

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Table 1. Clinical background

	Control $n = 20$	UC $n = 50$	p value
Age (SD)	54 (12)	48 (16)	0.11[a]
Gender (Female:Male)	12:8	25:25	0.45[b]
Mayo endoscopic subscore (MES) 0/1/2/3	—	16 (32%)/16 (32%)/13 (26%)/5 (10%)	—
Type of disease [*n* (%)] Proctitis/left-sided/pancolitis	—	9 (18%)/18 (36%)/23 (46%)	—
5-ASA, SASP [*n* (%)]	—	16 (32%)	—
PSL [*n* (%)]	—	0 (0%)	—
AZA, 6-MP [*n* (%)]	—	41 (82%)	—
Anti-TNFα antibody [*n* (%)]	—	0 (0%)	—

P values, [a] by unpaired *t* test and [b] by chi-square test. UC, ulcerative colitis; 5-ASA, 5-aminosalicylic acid; SASP salicylazosulphapyridine; AZA, azathioprine; 6-MP, 6-mercaptopurine.

Bloomington, IN) following usual colonoscopy preparation using polyethylene glycol (PEG). Reportedly, there is no difference in microbial diversity in biopsy samples taken from the ileum, ascending colon, descending colon, and rectum of patients with CD.[12] This study was approved by the Ethics and Medical Research Committee of the Kawasaki Medical School (CRB no. 3087). Written informed consent was obtained from each participant prior to enrollment.

DNA extraction and 16S rRNA sequencing. DNA extraction, preparation of a library of amplicons encoding the 16S rRNA gene, and sequencing were performed as previously described.[13] Samples were profiled by high-throughput amplicon sequencing with dual-index barcoding on the Illumina MiSeq platform (Illumina, San Diego, CA). The V3–V4 regions of the gene encoding 16S rRNA (460 bp) were amplified using PCR.[14] The PCR amplicons were purified using SPRI select beads (Beckman Coulter, Brea, CA). The DNA concentration of the purified amplicons was measured using a Quantus Fluorometer and the QuantiFluor® dsDNA System (Promega, Madison, WI); approximately equal amounts of DNA were pooled. The pooled sample was sequenced using the MiSeq Reagent Kit V3 (600 cycles) (Illumina) on the MiSeq system, according to the manufacturer's instructions. Sequence data were analyzed using the Quantitative Insights into Microbial Ecology (QIIME) pipeline (ver. 1.8.0).

Bioinformatics analysis. Sequence data processing, including quality filtering, chimera check, operational taxonomic unit (OTU) definition, and taxonomy assignment, was performed using QIIME ver. 1.9.0, USEARCH ver. 9.2.4, UCHIME ver. 4.2.40, and VSEARCH ver. 2.4.3. The singletons were then removed. The RDP classifier ver. 2.10.2 and the Greengenes database (published May, 2013) were used for taxonomic assignment of the acquired OTUs (97% sequence similarity).

α- and β-Diversities. The observed species, Chao1, and Shannon indices were calculated using the phyloseq package in R software. The β-diversity was estimated using the UniFrac metric to calculate the distances between samples using QIIME ver. 1.9.1. The β-diversity was visualized by principal coordinate analysis using R software and statistically analyzed by permutational multivariate analysis of variance (PERMANOVA) using QIIME ver. 1.9.1.

Statistical analysis. Values are presented as mean ± SD or median and 25–75% range, regardless of whether the data were normally or non-normally distributed. The categorical data were presented as counts with percentages and analyzed using the chi-squared test. The diversity and relative abundance of bacterial genera were compared among three groups with type of UC by Kruskal–Wallis analysis using SPSS software (ver. 26 for Windows; IBM Japan, Ltd., Tokyo, Japan) and compared between two groups using Welch's *t* test. The metagenome content predicted with PICRUSt was compared between the two groups using Welch's *t* test on STAMP software. $P<0.05$ indicated statistical significance.

Results

Patient characteristics. Fifty patients with UC and twenty healthy controls were enrolled in this study. The mean age (54 years in the UC group and 48 years in the control group) and the percentage of men were not significantly different between the two groups (Table 1).

In the UC group, majority (32%) of the patients had an MES of 0 and 1, 26% had an MES of 2, and 10% had an MES of 3. Therefore, 64% of patients were in remission. The percentage of patients with proctitis, left-sided colitis, and pancolitis were 18%, 36%, and 46%, respectively. A total of 41 (82%) patients were taking 5-aminosalicylates or sarazosulfapyridine, 16 (32%) were taking azathioprine, and none received anti-tumor necrosis factor alpha (TNF-α) therapy.

Ileum brush samples were obtained from all subjects, while sigmoid colon brush samples were not obtained from two patients due to severely inflamed mucosa.

Comparative analyses of the α- and β-diversity indices of UC patients and controls. UC patients displayed significantly reduced α-diversity in terms of Chao1 index, observed species, and the Shannon index compared to controls in both the MAM of the ileum and sigmoid colon (Fig. 1A and B). In the terminal ileum, the Chao1 indices were only significantly lower ($p = 0.039$) in the patients with MES 2 or 3 than in those with MES 0. However, there was no significant difference in other alpha-diversity indices assessed according to disease classification and the Mayo endoscopic score.

Although the β-diversity in weighted analysis, taking into account the number of leads contained in OTUs, was not significantly different between the two groups, the difference was recognized in unweighted analysis (Fig. 2).

Comparison of taxonomic composition at the genus level between UC patients and controls. The abundances of *Lactobacillus*, *Veillonella*, and *Pediococcus*, in the ileum were significantly higher in UC patients than in the controls. In contrast, the abundances of *Butyricicoccus*, *Ruminococcus*, *Lachnospiraceae*, and *Akkermansia* were significantly lower in the ileum of UC patients than in that of controls (Fig. 3A).

The abundances of *Lactobacillus and Pediococcus* were significantly higher, while those of *Butyricicoccus* and *Roseburia* were significantly lower, in the sigmoid colon of UC patients than in controls (Fig. 3B).

Comparison of taxonomic composition at the genus level among UC patients according to disease classification. In the terminal ileum, the abundance of organisms belonging to the

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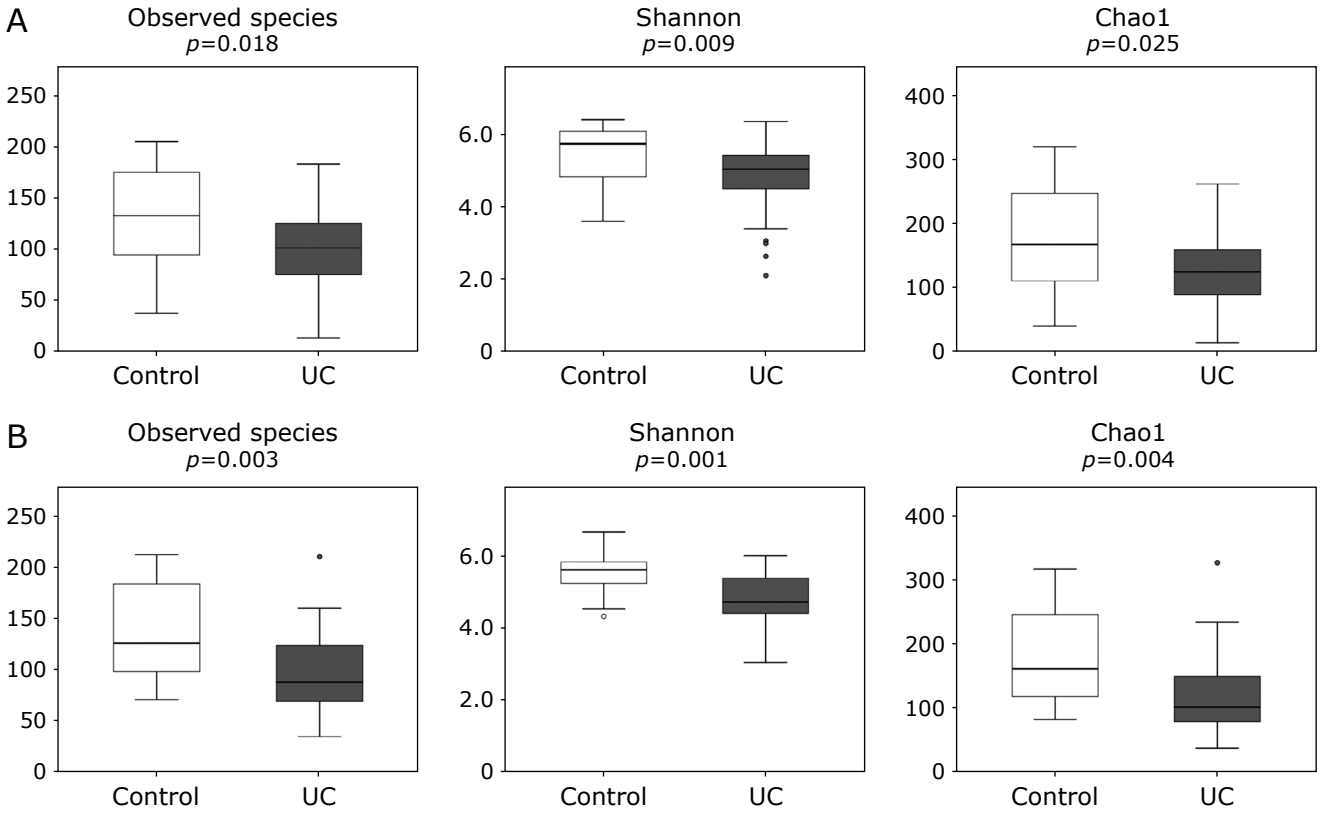

Fig. 1. Comparison of α-diversity indices of ulcerative colitis (UC) with controls. Comparison of α-diversity by Chao 1, Observed species, and Shannon index in the ileum (A) and in the sigmoid colon (B) was performed between controls (white square) and UC patients (black square).

genus *Ruminococcus* of the family *Lachnospiraceae* was significantly higher in left-sided colitis patients than in all other UC patients, while that of *Odoribacter* was significantly lower in left-sided colitis and pancolitis patients than in proctitis patients (Fig. 4).

Comparison of taxonomic composition at the genus level among UC patients according to MES. In the terminal ileum, the abundance of organisms belonging to the genera *Oscillospira* and the family *Ruminococcaceae* was significantly lower in UC patients with MES 2 or 3 than in those with MES 0. Similarly, the abundance of *Odoribacter* was significantly lower in UC patients with MES 2 or 3 than in those with MES 1. There was no significant difference in the abundance of these bacteria between the MES 0 group and the MES 1 group (Fig. 5).

Discussion

In this study, we performed screening of MAM in UC patients and found that *Odoribacter* abundance was significantly lower in left-sided colitis and pancolitis patients than in proctitis patients, and in patients of active phase (MES 2 or 3) than those in remission phase (MES 0 or 2) as evaluated by endoscopy. It is intriguing to hypothesize that *Odoribacter* in the ileum may suppress the spread of UC from the rectum to the oral side of colon and thus determine the severity of mucosal inflammation.

Odoribacter splanchnicus (formerly *Bacteroides splanchnicus*) is an anaerobic gram-negative bacterium that produces SCFAs, such as acetate, propionate, and butyric acid. SCFAs act directly on epithelial cells and modulate Treg responses. The reduction in *O. splanchnicus* abundance in IBD patients is thought to be related to a reduction in intestinal SCFA levels.[15,16] In a recent study conducted by Lima *et al.*,[17] who performed sequencing of immunoglobulin (Ig) A-coated microbiota, *O. splanchnicus* levels correlated with the clinical response to FMT in patients with UC as a transferable strain shaping mucosal immunity. The colonized *O. splanchnicus* in gnotobiotic mice correlated with the clinical response and induction of mucosal Tregs, IL-10, and SCFAs.[17] Moreover, *Odoribacter* was recently identified as an effective producer of isoallolithocholic acid, which has been shown to exert potent antimicrobial effects against gram-positive (but not gram-negative) multidrug-resistant pathogens such as *Clostridium difficile*. Novel bile acid biosynthetic pathways are enriched in the microbiome of centenarians.[18] In addition, outer membrane vesicles (OMVs) produced by *O. splanchnicus* could potentially exert beneficial immunoregulatory effects in the gut epithelium.[19] Therefore, *O. splanchnicus* can be characterized as a unique bacterium with more protective ability than other bacteria that are capable of inducing IL-10 and SCFAs.

Our study may suggest that ileal *Odoribacter* confers a protective role against the harmful effects of other bacteria in the colon; the reduction in *Odoribacter* levels results in increased UC activity and insufficient protection from disease propagation from the rectum to the right side of the colon. This hypothesis should be verified by investigating the relationship between disease activity and *Odoribacter* abundance in each part of the colon. In addition, since the mean abundance of *Odoribacter* in proctitis patients was very low, the precise function of this bacterium and *Odoribacter*-produced SCFAs should be further investigated using *in vivo* and *in vitro* model.

Compared to the MAM diversity in controls, the diversity of MAM was found to be significantly decreased in both the ileum and sigmoid colon in UC patients, consistent with previous results and systematic reviews.[8,20,21] It should be noted that significant differences were found in β-diversity with unweighted

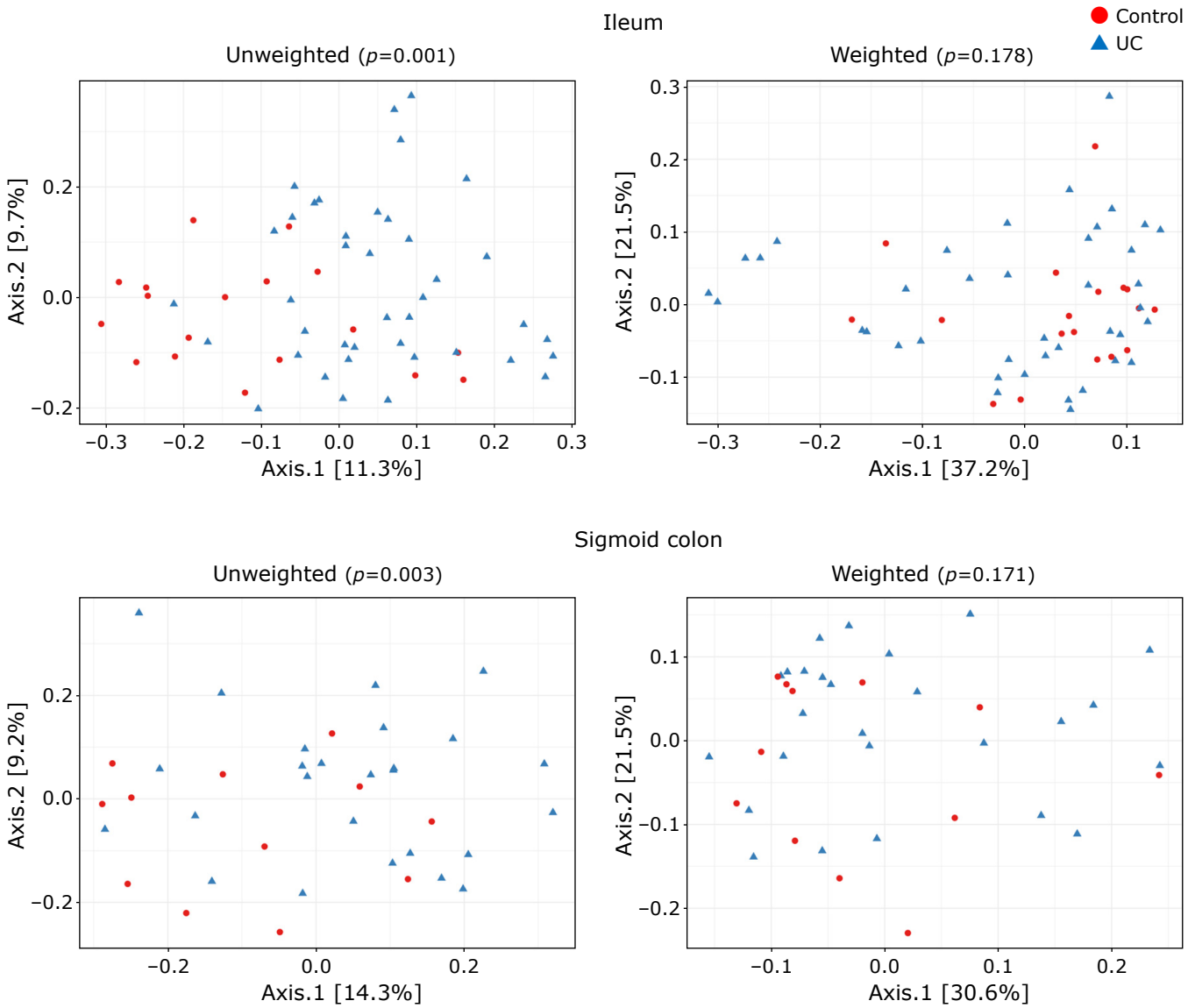

Fig. 2. Comparison of β-Diversity (Unifrac + Principal Component Analysis). Comparison of β-diversity in the ileum (upper) and the sigmoid colon (lower) was performed between the controls (circle) and the ulcerative colitis (UC) patients (triangle) by unweighted (right)- and weighted (left)-analysis.

but not with weighted analysis, suggesting that "minor species", including *Odoribacter*, have an influence on the pathogenesis of UC.

Moreover, in this study, the abundances of *Lactobacillus and Pediococcus* in the ileum and sigmoid colon and *Veillonella* in the ileum were increased in UC patients. *Lactobacillus* is a gram-positive aerotolerant anaerobe that produces lactic acid. Excessive lactic acid accumulation in the colon has been reported to be harmful to human health and must be assimilated into useful SCFAs.[22] *Veillonella* metabolizes lactic acid into propionic acid and acetic acid, and the increased lactic acid production by *Lactobacillus* may increase the abundance of this bacterium. On the contrary, the abundances of *Butyricicoccus* at both sites, *Ruminococcus* in the ileum, and *Roseburia* in the sigmoid colon were significantly lower in UC patients than in controls. These bacteria produce butyric acid, conferring anti-inflammatory effects through the production of IL-10 from dendritic cells and induction of Tregs. The reduction of butyric acid-producing bacteria in UC may aggravate colonic inflammation. Butyric acid is a major source of energy in the colonic epithelium and is metabolized via β-oxidation, which activates mitochondrial metabolism. The activation of mitochondrial metabolism increases the oxygen consumption of colonic epithelial cells, decreasing barrier oxygen concentration and resulting in an increase in the abundance of obligate anaerobic bacteria, such as those belonging to the phylum *Firmicutes*.[20]

Some limitations of this study include the relatively small number of subjects and the fact that more than two-thirds of the UC patients were in remission. The location of MAM sample collection also varied, which might have affected the results, although sample collection was performed at least 30 cm from the oral side of the ileocecal valve. Moreover, some sigmoid colon samples were collected from inflamed sites, which was unavoidable. Therefore, the results of MAM analysis in the sigmoid colon should be treated with caution. Since patients were taking different drugs, their MAM compositions may have been affected. In a previous study using brush samples obtained from patients with CD, anti-TNF-α therapy significantly affected the

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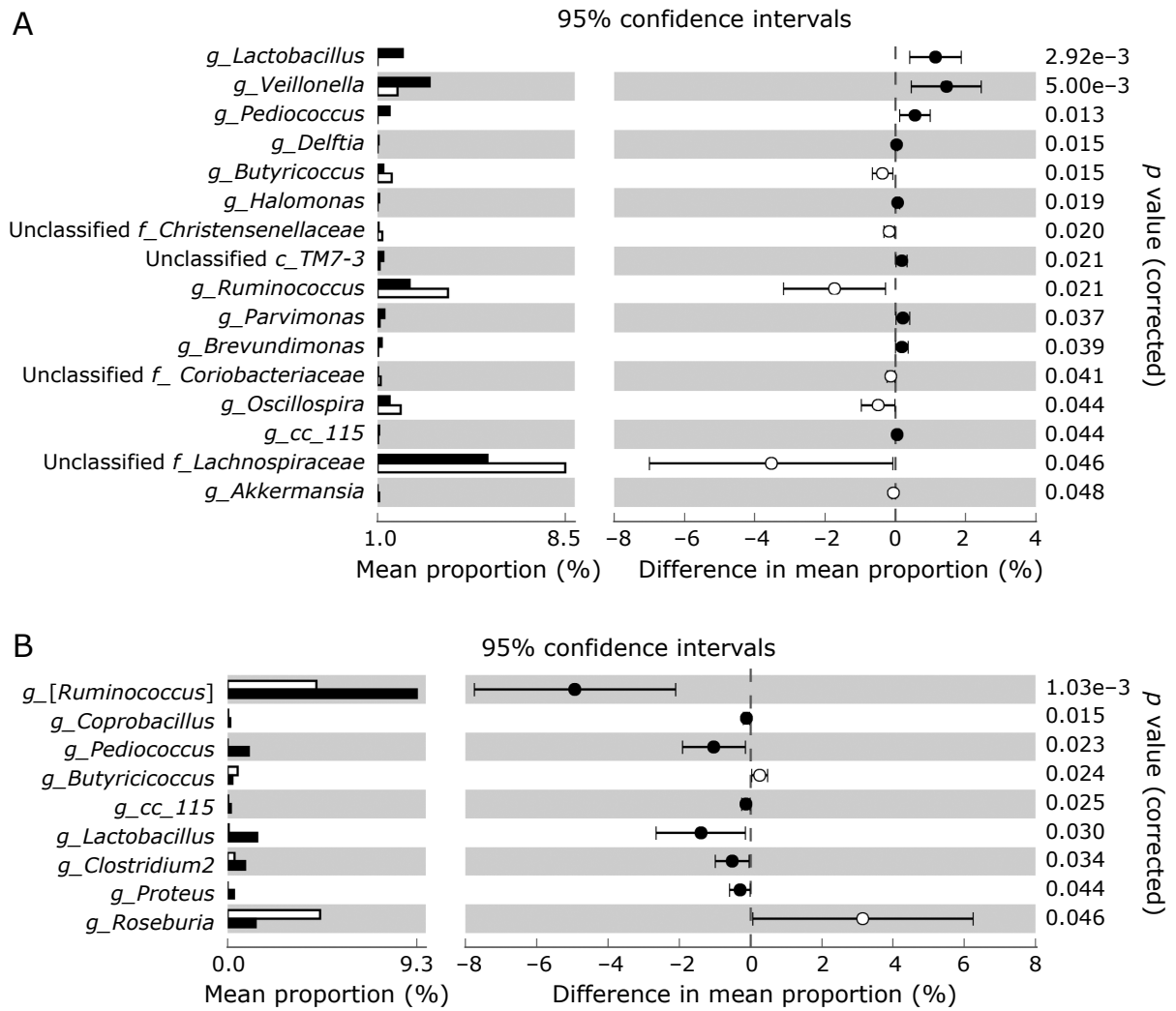

Fig. 3. Comparative analyses of the taxonomic composition of the microbial community at genus level between the patients (black) and the controls (white) in the ileum (A) and sigmoid colon (B).

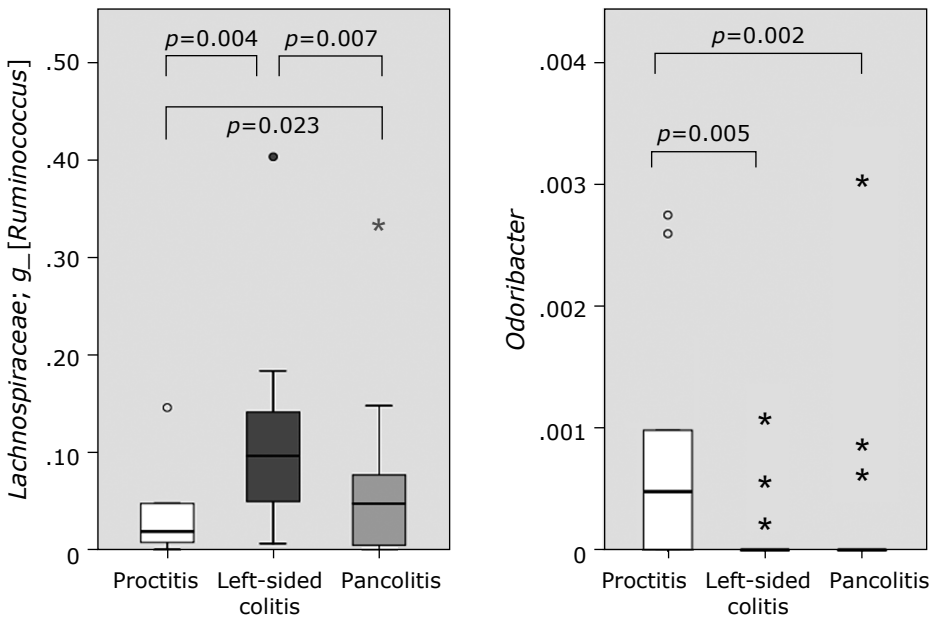

Fig. 4. The taxonomic composition at genus level in the ileum among the patients with proctitis, left sided and pancolitis.

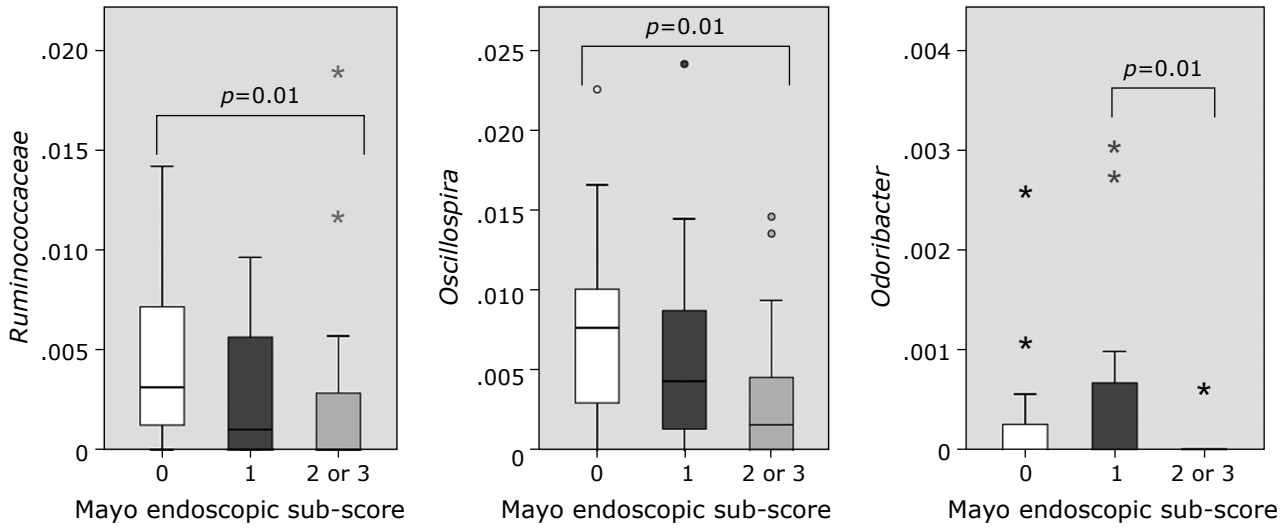

Fig. 5. Comparison of the taxonomic composition at genus level in the ileum among the tree groups according to the endoscopic severity. The endoscopic severity was evaluated by Mayo endoscopic subscore (MES): 0 = normal or inactive disease; 1 = mild disease (erythema, decreased vascular pattern); 2 = moderate disease (marked erythema, absent vascular pattern, any friability, erosions); 3 = severe disease (spontaneous bleeding, ulceration).

abundance of anti-inflammatory bacteria.[10] Therefore, patients treated with TNF-α were excluded from our study. However, the effects of other therapeutic drugs, including those for coexisting diseases, should be considered. Finally, PEG preparation is likely to affect microbiota composition and detection via the removal of luminal resident bacteria. However, our brush sample data suggest the strong attachment of MAM to the mucosa. These findings may play an important role in understanding the host-microbe interactions contributing to UC pathophysiology.

In conclusion, the reduction in abundance of of butyric acid-producing bacteria, especially *Odoribacter*, may play an important role in the pathophysiology of UC. Our findings may be valuable for informing the development of more efficient microbiome-based therapies for UC.

Author Contributions

OH and AS substantially contributed to the conception or design of the work, the acquisition, analysis, and interpretation of data for the work. MO, SF, HM, and EU contributed the acquisition, analysis of the sample. MO drafted the work, and OH, AS, RI, and YN revised it critically for important intellectual content. All authors finally approved the version to be published and agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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Abbreviations

CD	Crohn's disease
FMT	fecal microbiota transplantation
IBD	inflammatory bowel disease
IL	interleukin
MAM	mucosa-associated microbiota
MES	Mayo endoscopic subscore
PEG	polyethylene glycol
QIIME	Quantitative Insights into Microbial Ecology
SCFA	short-chain fatty acid
TNF-α	tumor necrosis factor alpha
Tregs	T regulatory cells
UC	ulcerative colitis

Conflict of Interest

YN received scholarship funds from Taiyo Kagaku Co., Ltd. and from EA Pharma Co. Ltd.; a collaboration research fund from Taiyo Kagaku Co., Ltd.; and received lecture fees by Mylan EPD Co., Takeda Pharma. Co. Ltd., Mochida Pharma. Co. Ltd., EA Pharma Co. Ltd., Otsuka Pharma. Co. Ltd., and Miyarisan Pharma. Co. Ltd. The present research was partly supported by these funds. Neither the funding agency nor any outside organization has participated in the study design or have any competing interests. These companies have approved the final version of the manuscript. All authors except for YN received no support, financial or otherwise, from any organization that may have an interest in the submitted work. There are no other relationships or activities that could appear to have influenced the submitted work.

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