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ORIGINAL ARTICLE

Microscopic Colitis Patients Possess a Perturbed and Inflammatory Gut Microbiota

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Abstract

Background Microscopic colitis (MC), an inflammatory disease of the colon, is characterized by chronic non-bloody diarrhea with characteristic inflammation and for some, collagen deposits in mucosal biopsies. The etiology of MC is unclear, although previous findings implicate luminal factors and thus the gut microbiome. However, the relationships between fecal microbiota and MC are relatively unexplored.

Methods Stool microbiota of MC ($n=15$) and healthy controls (HC; $n=21$) were assessed by 16S rRNA V4 amplicon sequencing and analysis performed in QIIME. Gut microbiota functions were predicted using Piphillin and inflammatory potential assessed using an in vitro HT29 colonocyte cell assay.

Results MC patient fecal microbiota were less diverse (Faiths index; $p<0.01$) and compositionally distinct (PERMANOVA, weighted UniFrac, $R^2=0.08$, $p=0.02$) compared with HC subjects. MC microbiota were significantly depleted of members of the *Clostridiales*, enriched for *Prevotella* and more likely to be dominated by this genus ($Chi^2=0.03$). Predicted pathways enriched in MC microbiota included those related to biosynthesis of antimicrobials, and sphingolipids, to glycan degradation, host defense evasion, and Th17 cell differentiation and activation. In vitro, exposure of cultured colonocytes to cell-free products of MC patient feces indicates reduced gene expression of IL-1B and occludin and increased GPR119 and the lymphocyte chemoattractant CCL20.

Conclusion MC gut microbiota are distinct from HC and characterized by lower bacterial diversity and *Prevotella* enrichment and distinct predicted functional pathways. Limited in vitro experiments indicate that compared with cell-free products from healthy fecal microbiota, MC microbiota induce distinct responses when co-cultured with epithelial cells, implicating microbiota perturbation in MC-associated mucosal dysfunction.

Keywords Dysbiosis · Gastrointestinal microbiota · Microscopic colitis · *Prevotella* · 16S rRNA sequencing

Introduction

Microscopic colitis (MC) is an inflammatory bowel disease (IBD) characterized by chronic non-bloody diarrhea with normal macroscopic findings on endoscopy, but with characteristic inflammation and/or collagen deposits in mucosal biopsies [1]. MC had previously been considered a rare condition; however, recent studies have found the disease to be equally prevalent as macroscopic IBD variants such as ulcerative colitis (UC) and Crohn's disease (CD), with a Danish incidence of 20,7 per 100,000 person-years [2]. MC is divided into two subtypes based on the histological findings of the colon: (1) lymphocytic colitis characterized by mucosal and lamina propria lymphocyte infiltration in the colon and (2) collagenous colitis with collagenous banding

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in lamina propria together with lymphocytic infiltration, although less than in lymphocytic colitis [1]. MC is characteristically associated with colonic mucosal infiltrates of cytotoxic CD8 +, Th1 and Th17 lymphocytes coupled with overexpression of associated inflammatory cytokines [3]. Although the etiology of MC is unclear, genetic risk factors such as IL-6–174 polymorphism and HLA-DQ2 haplotype have been implicated [3]. Further risk factors for MC include older age, female gender, smoking, prior treatment with Non-Steroidal Anti-Inflammatory Drugs (NSAID), proton pump inhibitors (PPI), and selective serotonin reuptake inhibitors (SSRI) [1, 4, 5]. Another potential risk factor is hormone therapy. A recent study by Burke et al. found increased risk of MC development for women receiving menopausal hormone therapy [6]; however, previous findings of Ohlsson did not find a correlation between MC and hormone therapy [7].

Gut microbiome perturbations have been linked to numerous diseases in recent years, in particular to inflammatory conditions of the gastrointestinal tract such as Crohn's disease and ulcerative colitis [8–10]. Recent studies have demonstrated that a relatively limited number of discrete distal gut microbiome structures exist in ulcerative colitis patients and relate to differences in clinical features of disease severity [11]. Moreover, the cell-free products of these distinct gut microbiomes induce significant different features of immune dysfunction in vitro [11], implicating gut microbiomes and their products in the clinical heterogeneity and immune dysfunction observed in IBD patients. While the gut microbiome could be an obvious potential contributor to MC, it remains largely unexplored with only few published studies [12–16]. Fischer et al. described a different gut microbiome of MC patients compared to healthy controls [12]. Morgan et al. found a significant difference between gut microbiota of patients with active or remission stages of MC [13]. This is further supported by the findings of Carstens et al., who also described a shift in specific bacterial families, comparable to that observed in CD and UC [16]. Millien et al. reported a significant enrichment of *Desulfovibrionales* in colonic biopsies in MC patients compared to healthy controls [14]. Lastly, Rindom Krogsgaards et al., observed that budesonide treatment increased gut microbiome bacterial diversity [15].

Previous findings that support a role for the gut microbiome in the pathogenesis of MC include a study by Järnerot et al. describing clinical and histopathological remission of collagenous colitis after fecal stream diversion through sigmoidostomy or ileostomy improvement [17] and clinical and histopathological remission in one patient following fecal microbial transplant (FMT) [18]. In addition, a case report also described remission of MC in a patient following temporary ileostomy; however, clinical relapse occurred following bowel reconstruction characterized by increased colonic mucosal inflammation [19]. Thus, we hypothesized that the gut microbiome of MC patients is compositionally distinct from that of healthy subjects and pathogenic. We sought to characterize the gut microbiome of MC patients and determine whether their functional features and products promote inflammatory responses characteristic of the disease.

Materials and Methods

Study Population and Sample Collection

Stool from Danish MC patients ($n = 15$) and healthy subjects, HC ($n = 21$) were collected in the period 2012–2015 as previously described [20, 21]. All MC patients had active disease (diarrhea ≥ 3 stools daily) upon sampling and had a confirmed MC diagnosis by histopathology. The HC group was sampled from subjects that were part of a screening program for genetic predisposition for colorectal cancer or hereditary non-polyposis colorectal cancer [20]. Stool samples from patients with Crohn's disease ($n = 21$) and ulcerative colitis ($n = 38$) were also included for comparisons with MC patients [20]. Only participants with no antibiotic use within 4 weeks prior to sampling were included. Stool samples were stored at -80 °C until processed for microbiota profiling. Basic demographics and clinical data of the participants are shown in Table 1.

DNA Extraction and Quantification

Total DNA was extracted from stool samples using a DNeasy PowerLyzer PowerSoil Kit (Qiagen, CA), following manufacturer's instructions with the following exception: Bead beating was performed following addition of lysis buffer C1 using the TissueLyzer II (Qiagen) at 5.5 m/s for 30 s. DNA was eluted in Solution C6 provided in the PowerSoil kit. DNA concentration was quantified using the Qubit 2.0 Fluorometer (Thermo Fischer Scientific) and the Qubit dsDNA BR assay kit (Thermo Fischer Scientific).

Amplicon Library Preparation and Sequencing

DNA concentrations were standardized to 10 ng/µl per reaction for 16S rRNA amplification targeting the V4 region using 515F and 806R primers with the following PCR settings: initialization (98 °C, 2 min), 30 cycles of denaturation at (98 °C, 20 s), annealing (50 °C, 30 s) and extension (72 °C, 45 s) and final extension (72 °C, 10 min) [22]. Triplicate amplifications were performed per sample and pooled amplicons purified using the SequalPrep Normalization Plate Kit (Thermo Fisher Scientific) according to the manufacturer's specifications, quantified using the Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific), and pooled at

Table 1 Study participants demographics

Demographics	Healthy controls	Microscopic colitis	Crohn's disease	Ulcerative colitis
No	$N=21$	$N=15$	$N=21$	$N=38$
Age (years), median	58 (± 10)*	72 (± 11)*	49 (± 19)	52 (± 18)
Gender, male	47% (10/21)	26% (4/15)	65% (13/21)	47% (18/38)
Active disease, n (%)	0 (0%)	15 (100%)	8/8 (13 unknown)	8/15 (23 unknown)
Lymphocytic colitis		$N=8$		
Collagenous colitis		$N=7$		

Statistical significant $p<0.01$, t test

Active disease: for MC: diarrhea (> 3 stools pr day), for CD and UC: diarrhea, stomach pain, bloody stools

equimolar concentrations. The amplicon library was concentrated using the Agencourt AMPure XP system (Beckman-Coulter), quantified using the KAPA Library Quantification Kit (KAPA Biosystems), and diluted to 2 nM. Equimolar PhiX was added at 40% final volume to the amplicon library prior to sequencing on an Illumina NextSeq 500 Platform.

Sequence Data Processing and Quality Control

Paired-end sequences of 153 base pairs were assembled using FLASH [23] with a 25 base pair overlap. Sequence reads from each lane were demultiplexed before concatenating and quality filtering using USEARCH [24], with a maximum expected error of 2. Quality-filtered reads were dereplicated and sorted by sequence count using USEARCH. Dereplicated sequences were clustered into Operational Taxonomic Units (OTUs) based on > 97% sequence identity with simultaneous removal of chimeric sequences using USEARCH. OTU sequences were aligned using PYNAST [25], and taxonomic classification was assigned using GreenGenes [26]. A phylogenetic tree was built using FastTree [27] and used to compute Faith's Phylogenetic Diversity and UniFrac distances using on OTU table multiply rarefied to 25,535 sequences per sample.

Microbial Functional Capacity Estimate

Conserved functional traits of the gut microbiome were predicted based on 16S rRNA profiles using Piphillin [28] which produces an in silico metagenome.

Cell-Free Fecal Extract Preparation

Cell-free extracts were prepared from fecal samples of six subjects (MC = 3, HC = 3) with sufficient sample remaining as previously described [29]. In brief, 1 g/ml w:v of stool was added to pre-warmed phosphate-buffered saline with 20% fetal bovine serum, vortexed, incubated at 37 °C for 10 min, centrifuged at 14000 rpm for 1 h, and filter sterilized initially through a 0.45-µm and subsequently a 0.22-µm filter. Fecal cell-free extracts were stored at −80 degrees until assayed.

HT-29 Epithelial Cell Assay

Human colon adenocarcinoma HT-29 epithelial cells were seeded at a confluent density in 96-well plates in fresh McCoy's 5a media (Thermo Fisher Scientific) supplemented with 10% heat-inactivated fetal bovine serum (FBS; USA Scientific) and 100 U/ml penicillin–streptomycin (Life Technologies) 24 h before exposure to fecal cell-free extracts. Cells were treated with 25% v:v cell-free extracts for 24 h. Total RNA was isolated with RNAqueous-Micro Kit (Thermo Fisher Scientific) and contaminant DNA digested with DNase I (Sigma) for 20 min at 37 °C. First-strand cDNA was synthesized using 300 ng RNA with High-Capacity RNA-to-cDNATM Kit (Thermo Fisher Scientific) according to the manufacturer's instructions. Expression of genes involved in response to microbes and their products (*TLR4, β-defensin-1, PPARγ, GRP119*) as well as those governing lymphocyte recruitment (*CCL20*), inflammation (*TGF-β, IL-1β*) and epithelial integrity (*Occludin*), were assessed by real-time quantitative PCR (RT-qPCR) to provide a holistic view of pathways induced by cell-free fecal microbiomes (*primers listed in Table S2*). RT-qPCR was performed in triplicate per sample using SYBR Green master mix (Life Technologies) in the QuantStudio™ 6 Flex Real-Time PCR System (Applied Biosystems). RT-qPCR conditions were as follows: 50˚C for 2 min, 95˚C for 10 min (1 cycle); 95˚C for 15 s and 60˚C for 1 min (40 cycles); and a final melting curve cycle of 95˚C for 15 s, 60˚C for 1 min and 95˚C for 15 s. Gene expression was normalized to β-actin expression and relative fold change in expression determined by the $2^{-\Delta\Delta CT}$ (where C_T is threshold cycle) method using PBS-exposed cells as control.

Statistical Analysis

Bacterial alpha diversity indices (Shannon, observed species, Faiths phylogenetic diversity) were calculated using Quantitative Insights Into Microbial Ecology (QIIME) and

between-group comparisons performed using Mann–Whitney test (GraphPad Prism). Beta diversity was calculated using weighted and unweighted UniFrac distance matrices and visualized using principle coordinates analysis (PCoA) using Emperor in QIIME [30]. To determine factors that significantly ($p < 0.05$) explain variation in bacterial beta diversity, permutational multivariate analysis of variance (PERMANOVA) was performed (*Adonis* in R). To account for differences in data distribution at the taxon level, a three-model approach (Poisson, negative binomial, and zero-inflated negative binomial mixed-effect models) corrected for multiple testing ($q < 0.10$) was employed for between-group taxon comparisons as previously described [31]. Mann–Whitney test adjusted for multiple testing ($q < 0.15$) was used to determine specific predicted functional pathways differentiating the microbiota of MC and healthy subjects. Beta diversity was calculated using Bray Curtis distance matrix and visualized using principle coordinates analysis (PCoA) using Emperor in QIIME [30]. Difference in gene expression of colonocytes induced by fecal cell-free extracts from MC and HC subjects was evaluated using Mann–Whitney test, and gene expression signature between groups was analyzed with PERMANOVA and visualized by PCoA.

Results

MC Patients Exhibit a Distinct Diversity-Depleted Gut Microbiota

MC patient fecal gut microbiota alpha diversity was compared with that of IBD patients (ulcerative colitis (UC; $n = 38$) and Crohn's disease (CD; $n = 21$) and healthy controls (HC, $n = 21$). MC patients exhibited a significantly less diverse gut microbiota than HC subjects (Mann–Whitney; $p < 0.01$), to a degree comparable to that observed in UC and CD patients (Kruskal–Wallis with Dunn's test of multiple comparisons, $p > 0.05$; Fig. 1a). Loss of diversity in MC microbiota was primarily driven by reduced taxonomic richness (Student's t test, $p = 0.0003$, Fig. 1a, b). No significant difference in alpha diversity was observed between MC subtypes (Mann–Whitney, $p > 0.05$). Because MC patients were significantly older than the HC population (mean ± SD 71 y ± 12 versus 58 y ± 10; Student's t test, p value < 0.01), we tested age as a confounding factor by Spearman's rank test, but found no significant correlation between age and alpha diversity in our cohort ($r = -0.18$, $p = 0.27$).

MC patients possessed a significantly distinct gut microbiota composition from that of HC both in terms of types of microbes present (Unweighted UniFrac, PERMANOVA, $R^2 = 0.11$, $p < 0.01$; Fig. 1c) and their relative abundance (Weighted UniFrac, PERMANOVA, $R^2 = 0.8$, $p = 0.02$; Fig. 1d). Subject age, gender, and year of sample collection also significantly related to variance in gut microbiota composition (Unweighted UniFrac; PERMANOVA; age $R^2 = 0.04$, p value = 0.04; gender, $R^2 = 0.05$, p value = 0.01; year of sample collection, $R^2 = 0.09$, p value < 0.01). Since the year of sample collection co-varied with gut microbiota composition, we determined whether this observation related to sample integrity. A comparison of DNA concentrations of samples collected in each year indicated no significant between-year difference (Mann–Whitney, $p = 0.32$), suggesting that additional factors associated with these years may account for this observation. Sample collection was conducted without changes during the inclusion period.

We recently demonstrated that distinct pathogenic gut microbiota structures, characteristically dominated by a specific bacterial taxon, relate to variance in immune phenotypes and clinical outcomes in a number of patient populations [11, 29, 32]. To determine whether this phenomenon extended to MC patient populations both the dominant bacterial taxa for each stool sample, i.e., the taxon that accounted for the greatest proportion of total 16S rRNA sequence reads for that sample, and the degree of taxon dominance, i.e., the proportion of total sequence reads attributable to the dominant taxon was determined for each subject. Across the entire cohort samples were primarily dominated by *Bacteroides, Prevotella*, or *Other* (samples were categorized as *other* if dominant taxon was only detected in a single sample). A comparison of MC and HC groups based on dominant taxon as a sample classifier indicated that MC microbiota were significantly more likely to be *Prevotella*-dominated than in HC subjects (Chi^2 $p = 0.03$; Fig. 2a). Dominant taxa also explained a substantial portion of variation in microbiota composition (PERMANOVA; Weighted UniFrac, $R^2 = 0.56$, $p < 0.01$, Fig. 2b).

At the taxon level, a total of 112 OTUs significantly differed in relative abundance between MC and HC subjects, 77 of which were depleted in MC patients and 35 significantly enriched (Fig. 2c, Table S4). Consistent with initial observations, MC patients exhibited significant enrichment of *Prevotella* as well as *Veillonella* and *Gammaproteobacteria* and a concomitant depletion of *Blautia, Dialister, Butyricomonas, and Clostridiales* particularly *Ruminococcaceae* (Fig. 2c). Multiple distinct *Prevotella* OTUs were highly abundant in MC patients, accounting for up to 71% of the total microbiota in some patients. The most significantly enriched OTU belonged to the *Prevotella* genus and exhibited a more than tenfold relative enrichment compared with HCs (OTU_3). All significantly enriched *Prevotella* OTUs were assigned as *Prevotella copri* or *Prevotella stercorea* using the GreenGenes database.

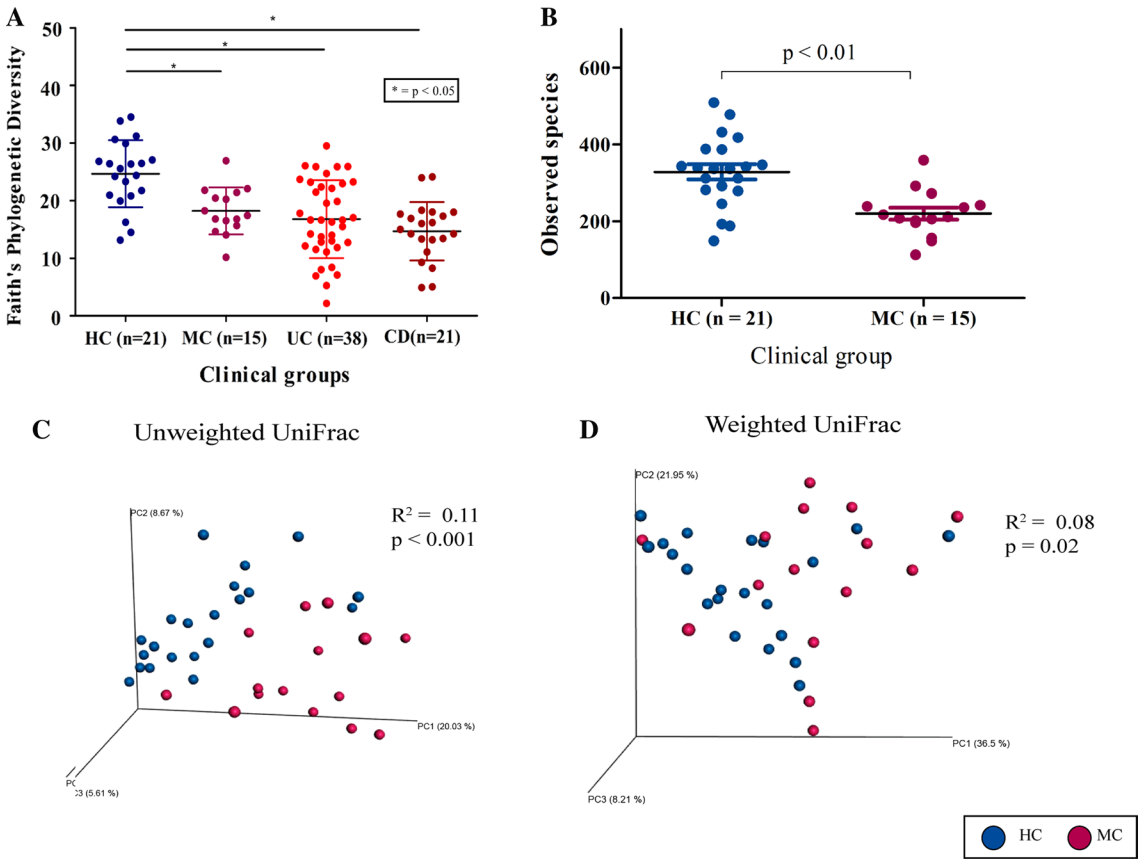
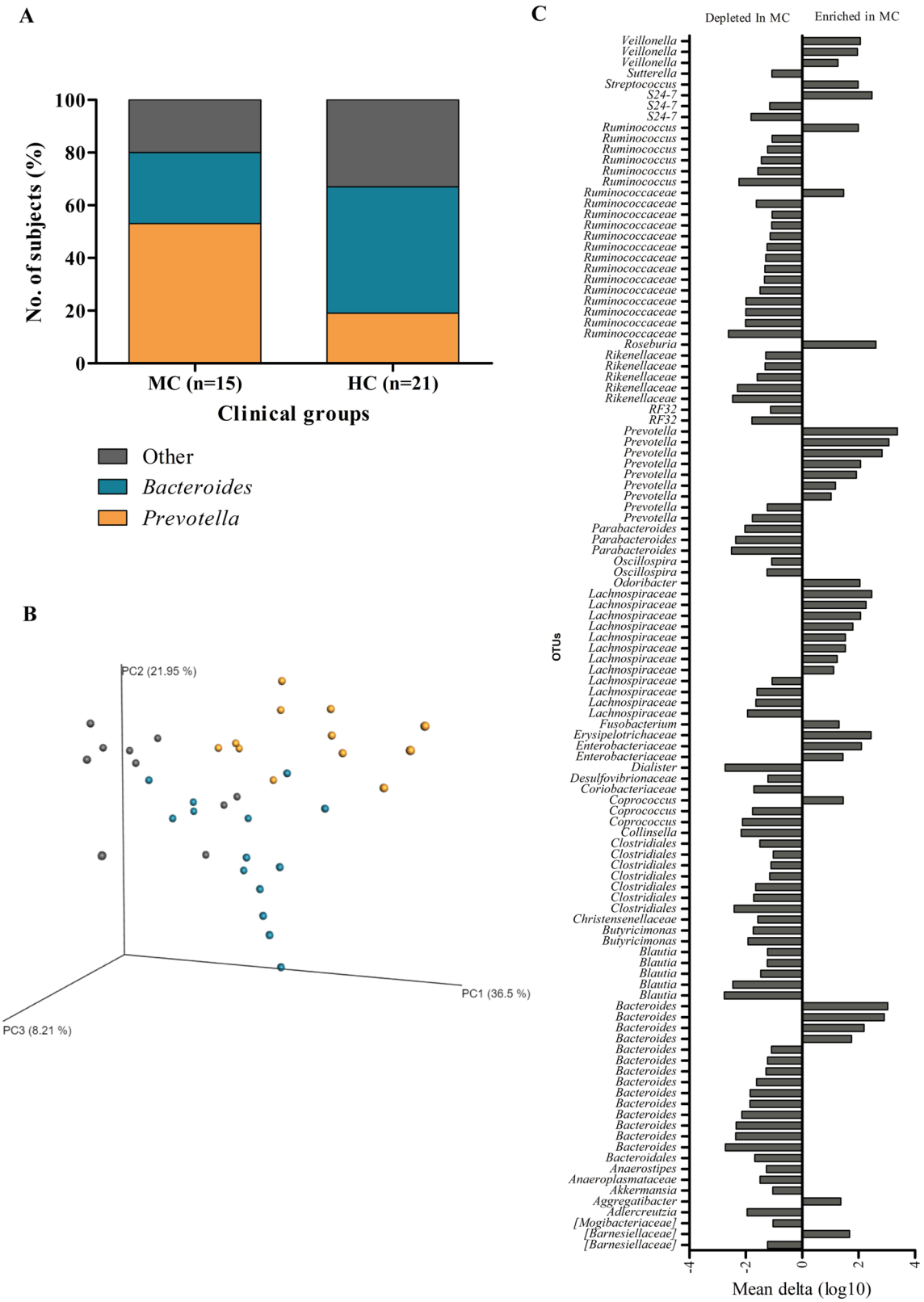


Fig. 1 Gut microbiota diversity is significantly reduced in IBD patients compared with healthy subjects. **A** Patients with microscopic colitis (MC) exhibit significantly reduced gut microbiota bacterial diversity (Faiths Phylogenetic Diversity, Mann Whitney, $p < 0.01$) compared to healthy (HC) subjects. MC diversity was not significantly different from that of ulcerative colitis (UC) and Crohn's disease (CD) patients (Kruskal–Wallis with Dunn's test of multiple comparisons, $p > 0.05$). **B** MC gut microbiomes exhibit reduced bacterial richness (observed species) compared with HC subjects ($p < 0.05$, Student's t test). **C**, **D** Bacterial community composition significantly differed between (MC) patients and healthy (HC) subjects. Principal coordinate analysis of Unweighted UniFrac (1.C) and Weighted UniFrac (1.D) distance shows compositional dissimilarity between clinical group ($p < 0.05$; PERMANOVA)

MC Gut Microbiome Is Functionally Distinct and Increases Colonic Epithelial Inflammation In Vitro

Given the significant differences in MC patient gut microbiomes, we hypothesized that these microbiomes would exhibit distinct functional traits related to disease pathogenesis. Piphillin was used to predict conserved bacterial functional pathways based on 16S rRNA data and the subsequent predictions of microbiome functional capacity were compared across MC and HC groups. As expected, the genes predicted to be encoded by MC and HC gut microbiomes were significantly distinct (Bray Curtis, $R^2 = 0.09$, $p = 0.018$; Fig. 3). Of the 297 predicted KEGG pathways (Kyoto Encyclopedia of Genes and Genomes), 123 (~40%) were significantly enriched in MC patients (Mann–Whitney test; q value < 0.015). MC-enriched pathways included those involved in triggering host immunity, particularly IL-17 signaling and Th17 cell differentiation, antigen processing and presentation, and NOD-like receptor signaling. Pathways involved in antimicrobial biosynthesis and resistance as well as metabolism of glutathione, an antioxidant, and glycan degradation, fatty acid, steroid biosynthesis, and bacterial secretion systems were also increased in MC patients. A relatively small number of bacterial-predicted pathways were decreased in MC patients relative to HC, the majority of which were associated with response to viruses *(Table S5).* These data, based on conserved functional traits of bacteria detected in MC patients, indicate enhanced inflammatory potential and a large number of pathways plausibly related to pathogenicity.

Given the significant difference in microbiome composition and enrichment of bacterial pathways involved in inflammatory processes in MC patients, we considered that the products of their fecal microbiome may promote epithelial dysfunction and inflammation. To assess this, we treated colonic epithelial cells with cell-free extracts of MC ($n = 3$) and HC ($n = 3$) patient feces and examined gene expression


A
100
80
60
40
20
0
No. of subjects (%)
MC (n=15)
HC (n=21)
Clinical groups
Other
Bacteroides
Prevotella
B
PC2 (21.95 %)
PC1 (36.5 %)
PC3 (8.21 %)
C
Depleted In MC
Enriched in MC
OTUs
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Veillonella
Veillonella
Sutterella
Streptococcus
S24-7
S24-7
S24-7
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Bacteroidales
Anaerostipes
Anaeroplasmataceae
Akkermansia
Aggregatibacter
Adlercreutzia
[Mogibacteriaceae]
[Barnesiellaceae]
[Barnesiellaceae]
-4
-2
0
2
4
Mean delta (log10)

◂**Fig. 2** **A** Dominant taxon distribution differed between microscopic colitis (MC) and healthy controls (HC). *Prevotella* was significantly more frequently observed as dominant taxon in MC patients, $p = 0.03$ (Chi^2). **B** Bacterial community composition and not membership alone were associated with dominant taxon, principal coordinates analysis with Weighted UniFrac ($p < 0.001$, PERMANOVA). **C** Individual distribution of OTUs that were significantly enriched or depleted ($q \leq 0.1$) in relative abundance in MC compared to HC measured in mean difference (Log10) in relative abundance of specific bacterial taxa significantly enriched (right) or depleted (left) in MC compared to HC

of a number of genes, permitting assessment of epithelial response to gut microbiome products. Although sample size was small which limits statistical detection of significant differences between groups, MC fecal extracts appear to induce a distinct gene expression signature compared to that of HC (Fig. 4a and b). Principal coordinates analysis depicts a clear separation of gene expression profiles between groups; however, it was not statistically significant *(PERMANOVA; Euclidean*; $R^2 = 0.47$, $p = 0.1$). Specifically, a trend toward reduced *IL-1β* and *Occludin* and increased *GPR119* and *CCL20* expression (Fig. 4b) was observed; however, it was not statistically significant (Mann–Whitney test: $p > 0.05$).

Discussion

The healthy gut microbiome typically houses several hundred microbial species encoding approximately two million genes [33]. Consistently, chronic inflammatory disorders, particularly those that fall under the IBD umbrella, have been associated with reduced bacterial diversity, loss of microbial function, and metabolic dysfunction in the gut [11, 34, 35]. The microbiota of MC patients are largely unexplored with only a few published studies primarily describing changes in composition [12–15]. Consistent with those reports, we observed that MC patients have a significantly distinct gut microbiome compared to HC, characterized by reduced diversity and a distinct repertoire of bacteria. Bacterial depletions in MC gut microbiomes included several genera known to be important for intestinal homeostasis such as *Clostridiales* [36], suggesting that loss of such microbes and their influence on immune function is characteristic and perhaps contributory to MC symptomatology.

A Swedish study of 17 subjects has previously described a depletion of *Akkermansia muciniphilia* and an enrichment of *Bacteroides* and *Prevotella* in the gut microbiome of MC patients compared with healthy controls [12], while a study by *Millien* et al. investigated microbiome features of colonic biopsies of MC patients compared to healthy subjects and reported a significant enrichment of the potentially proinflammatory family *Desulfovibrionales* [14]. A recent population-based study by Nielsen et al. found an increased risk of MC after *Campylobacter concisus* infection [37]. However, we did not detect significant enrichment of any species within the order of *Campylobacterales* in our 16S rRNA data.

A recent study by Morgan et al.[13] did not find a significant difference in alpha diversity between MC, healthy subjects, and functional diarrhea, but did observe a significant difference between patients with active or remission stages of MC. Consistent with our observations, the authors observed a significant increase in the relative abundance of *Haemophilis parainfluenza*, a member of the *Gammaproteobacteria* and *Veillonella parvula*, and other unclassified *Veillonella* [13], suggesting that conditions that permit expansion and activities of these opportunistic pathogens play a role in MC. Carstens et al. reported gut microbiota differences between active and remission collagenous colitis patients, and consistent with our findings, they found a significant decrease in the *Ruminococcaceae family, Rikenellaceae family, Clostridiales*, and *Akkermansia* [16]. Carstens et al. further compared CC microbiome to CD and UC and found that the decrease in *Ruminococcaceae* was present in all three diseases [16]. The IBD gut microbiota are characterized by a decreased diversity and loss of significant anti-inflammatory firmicutes such as *Roseburia* and increase in *Proteobacteria* and *Veillonella* [38]. Many of these dysbiotic traits are also present our MC cohort and might suggest that similar microbiological mechanisms are involved in pathogenesis of both MC and IBD.

These data coupled with our observations suggest that perturbations to the luminal microbiome influence gut epithelial gene expression and thus the microbial colonization landscape at the mucosal surface. Collectively these activities may thus contribute to MC pathogenesis. The finding by Rindom Krogsgaards et al., that treatment with budesonide (standard management of MC) increased gut microbiome bacterial diversity promoting an assemblage more reflective of that of healthy controls [15], further supports this concept and suggests that microbiome-targeted therapies may offer a novel approach for management or reversal of disease.

Our finding that *Prevotella* is significantly enriched in MC patient gut microbiomes is consistent with observations in others although not all investigated MC populations [12] and implicates this genus in MC pathogenesis. *Prevotella* are Gram-negative members of the *Bacteriodetes* phylum commonly considered commensals inhabitants of the human microbiome as they are found at several body sites in healthy humans [34, 39]. However, in recent years, several studies have demonstrated increased abundance of specific *Prevotella* species in patients with chronic inflammatory disorders such as IBD, rheumatoid arthritis, and periodontitis [39]. Specifically *P. copri* expansion has been associated with a number of chronic autoimmune diseases [39] such as rheumatoid arthritis (RA) [40, 41] and a protein expressed by this

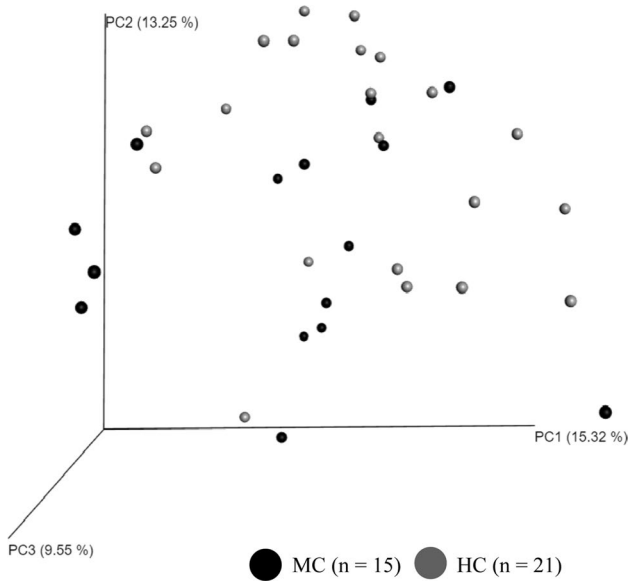

Fig. 3 PCoA plot of predicted gut microbiome functions (KEGG pathways) of microscopic colitis, MC ($n=15$, black), is significantly different from healthy controls, HC ($n=21$, gray), Bray Curtis, $R^2=0.09$, $p=0.018$

species, *Pc-p27*, has been shown to induce IFN-γ secretion from peripheral blood polynuclear cells of RA patients [42]. Moreover both new-onset and chronic RA patients exhibit elevated concentrations of antibodies to either Pc-p27 or the whole organism [42]. These findings are especially interesting, because MC patients have a higher frequency of autoimmune conditions, such as celiac disease and RA [1, 43, 44], emphasizing the importance of further exploring the clinical relevance of *P. copri* in MC.

Evidence that *P. copri* may enhance colonic inflammation comes from murine colitis models. In one such model, *P. copri*-colonized mice exhibit greater epithelial inflammation and weight loss compared to mice colonized with *Bacteroides thetaiotaomicron* [40], associated with increased IFN-γ production of Th1 cells in lamina propria. In addition, increased human serum levels of IFN-γ as well as IL-12 have been positively correlated with *P. copri*-specific IgA antibody in rheumatoid arthritis patients [42]. Interestingly, enhanced transcription levels of IFN-γ and IL-12 have been observed MC, and these cytokines have the capacity to

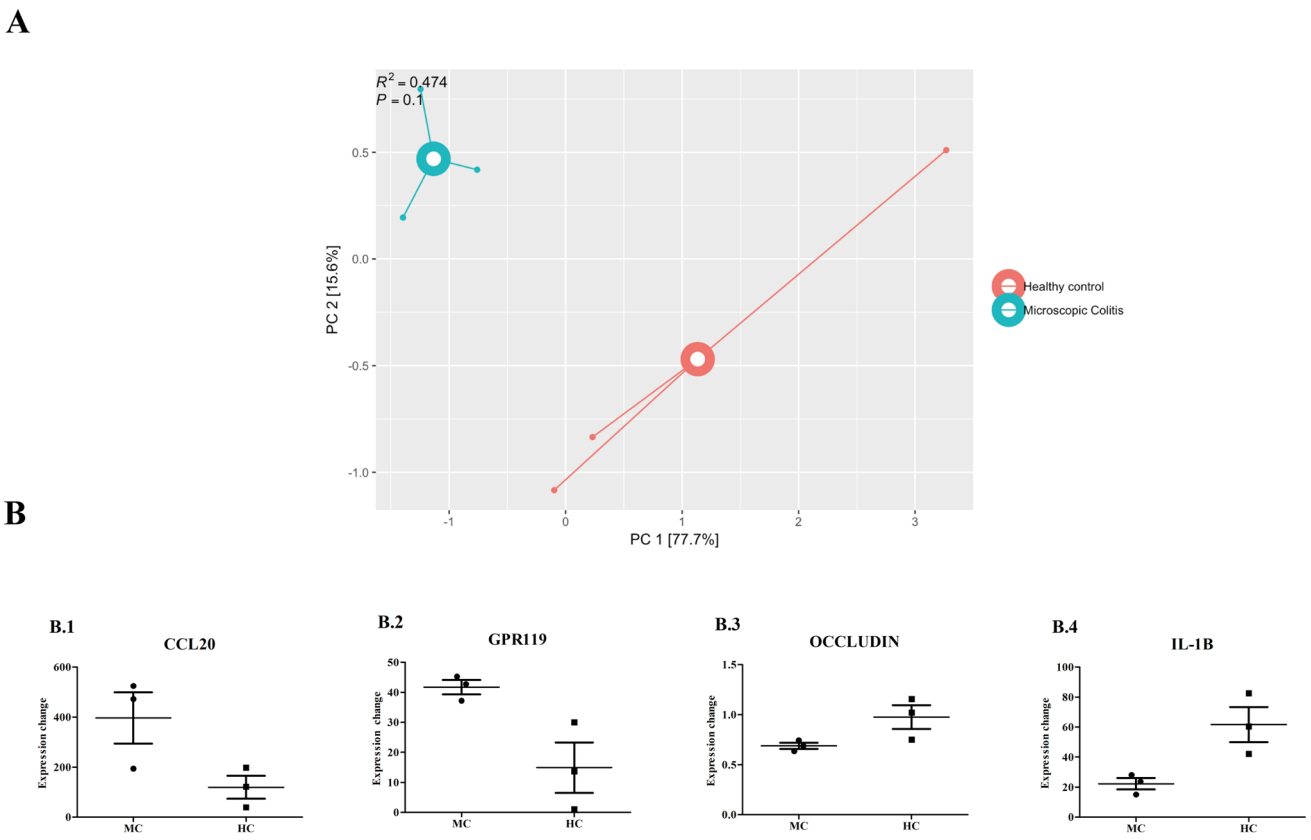

Fig. 4 **a** Difference in gene expression signature of HT29 cells exposed to cell-free extracts from MC ($n=3$) compared to HC ($n=3$). Principal coordinate analysis plot of Euclidean distance shows separation of gene expression signatures between groups; however, it did not reach statistical significance (PERMANOVA; $R^2=0.47$, $p=0.10$). **b** Gene expression of HT29 cells challenged with cell-free extracts from MC patients ($n=3$) compared to HC ($n=3$). **B.1** CCL20, **B.2** GPR119, **B.3** Occludin, **B.4** IL-1B. Mann–Whitney; $p>0.05$ for all tested gene expressions

convert Th17 cells into Th1 cells [3, 39], thus offering a plausible role for *P. copri* in MC development.

We also observed co-enrichment of several other opportunistic pathogens in *Prevotella*-dominated MC microbiomes, including *Veillonella* [13], *Roseburia* [12], and *Enterobactericeae*, thus indicating an overall shift in pathogenic potential of the enteric microbiome of MC patients and loss of species that promote immune and barrier integrity in the gut. In agreement with an earlier observation [12] we observed loss of *Akkermansia,* a genus known to increase epithelial barrier function [45], *Clostridiales*, especially *Ruminococcus* and additional members of *Ruminococcaceae*, which can induce T regulatory cells [36]. Finally, depletion of *Blautia* was observed in MC, and species within this genus have been shown to promote anti-inflammatory properties in in vitro experiments [46] and in host-versus-graft-disease, colorectal cancer, inflammatory pouchitis, and liver cirrhosis [47]. MC microbiomes were enriched for several KEGG pathways associated with immune activation, evasion of immune defenses, and production of antimicrobial compounds. This included enrichment of IL-17 signaling pathway and Th17 cell differentiation pathways which have previously been implicated in MC pathogenesis [3]. Consistent with these predictions, exposure of colonic epithelial cells exposed to the cell-free products of MC patients trended toward an increased *CCL20* expression, a chemokine that selectively recruits lymphocytes, including Th17 cells [48]. We also detected a trend of enhanced expression of *GPR119* following stimulation with MC microbiome products. Known ligands for this receptor include phospholipids and fatty acid amides, and this G-protein-coupled receptor is known to regulate release of anti-inflammatory peptides [49]. Of note, the MC gut microbiome was predicted to be enriched in pathways for fatty acid biosynthesis. Although the role of *GPR119* in IBD pathogenesis remains unclear, our observations suggest that its expression may be influenced by the lipid products of MC-associated gut microbiomes and thus play a role in regulating MC-associated inflammation. A trend toward decreased *Occludin* expression after exposure to MC microbiome products further indicates the capacity to decrease epithelial integrity. Lastly, an unexpected trend of decreased IL-1β was observed after exposure to MC microbiome products. When combining all investigated gene expressions into a gene expression signature PCoA depicts a clear separation between MC and HC groups; however, it did not reach statistical significance. The cell-free extract assay was conducted on a low sample size, and the preliminary results should be repeated with additional samples from independent cohorts.

The predicted functions of the MC gut microbiome were significantly different compared to HC, with increased pathways related to antimicrobials and antibiotic resistance, perhaps reflective of past perturbing influences on the MC gut microbiome. Predicted increases in fatty acid biosynthesis and metabolism together with increased secondary metabolite biosynthesis and sphingolipid biosynthesis and metabolism pathways indicate that the MC microbiome likely produces a distinct range of bioactive products responsible for the altered epithelial responses observed in vitro. Sphingolipids are integral human cell membrane lipids [50] and involved in the control of apoptosis, as well as the differentiation and proliferation of intestinal cells [50]. Disrupted sphingolipid metabolism promoting inflammation has been described for IBD [50]. Franzosa et al. found significantly increased concentrations of ceramide and sphingomyelin in CD and UC patients [10]. Ceramide can elicit proinflammatory responses and decrease intestinal barrier function [50] and is the end product of a pathway enriched in the MC gut microbiome (ko00603). Thus, dysregulation of sphingolipid signaling represents one potential pathogenic strategy of MC microbiomes. Additional pathways potentially contributing to pathogenic properties of the MC microbiome include glycan degradation, leading to mucin degradation and disruption of barrier function and increased capacity for degradation of glutathione, a potent antioxidant, leading to higher levels of oxidative stress—also a strong microbial selective pressure.

Our findings, though based on relatively small sample size, further support the role of the gut microbiota in pathogenesis of MC, suggesting that expansion of pathogenic bacteria, including *Prevotella* species and concomitant depletion of commensal taxa, contribute to associated colonic inflammation. A number of limitations to our study must be acknowledged, including the observed between-group age difference, in which MC patients were significantly older than HC subjects, although it proved non-significant for alpha diversity. While age may influence gut microbiome beta diversity, the factors explaining the greatest degree of variance in microbiota composition were clinical groups and dominant taxon. The mean age of MC diagnosis is 66 years, thus rendering it possible that age-dependent gut microbiome changes are a risk factor for MC development. It should be noted that MC consists of two subtypes, and possible differences in gut microbiome composition between subtypes are not assessed in the current study due to sample size, but should be investigated further. Furthermore, the small sample size of our cell-free extract assays renders the results preliminary and should be confirmed in a larger cohort. Lastly, our study is based on 16S rRNA V4 biomarker gene sequencing, which only detects bacteria and offers limited resolution at the species level. Future microbiome studies in larger cohorts are required, to capture perturbations to the composition of the whole gut microbiome (including mycobiome and virome), its functional gene capacity, and bioactive molecular productivity. Coupling of these studies

with model systems will permit gut microbiome contributions to microscopic colitis to be more fully elucidated.

Conclusion

The findings of this study support a role of the gut microbiota in pathogenesis of microscopic colitis. Data indicate that expansion of opportunistic pathogenic bacteria associated with inflammatory diseases, including *Prevotella* at the expense of anti-inflammatory commensal species, may contribute to inflammation and symptomology in these patients. Predicted functional gene capacity of MC microbiomes is distinct, and in vitro experiments indicate that their cell-free products induce a different response from epithelial cells. Thus, gut microbiome-targeted therapies, including dietary and live microbial interventions, may offer a novel approach for management or reversal of disease.

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Declarations

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. Stool samples used in this study were collected in clinical studies approved by the Regional Ethics Committee of Northern Jutland, Denmark (N-20130070).

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