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eHealth: Disease activity measures are related to the faecal gut microbiota in adult patients with ulcerative colitis

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ABSTRACT

Background/Aim: Microbial dysbiosis in inflammatory bowel disease (IBD) is poorly understood. Faecal samples collected for the purposes of microbiota analysis are not yet a part of everyday clinical practice. To explore associations between faecal microbiota and disease activity measures in adult IBD patients, for the purpose of possibly integrating microbiota measures in an existing IBD eHealth application for disease-monitoring.

Methods: We collected faecal samples from adult IBD patients for one year while they were home-monitoring for disease activity, using faecal calprotectin (FC) and the Simple Clinical Colitis Activity Index (SCCAI). Faecal samples were analysed in two different ways: commercially available test consisting of 54 pre-determined bacterial markers (DNA test) and 16S rRNA gene sequencing (16S-seq). Univariable linear mixed effect models were fitted to predict disease scores using normalised relative abundances as fixed effects.

Results: Seventy-eight IBD patients provided a total of 288 faecal samples for microbiota analysis. Two hundred and thirty-four of the samples were from patients with ulcerative colitis (UC). *Peptostreptococcus anaerobius* was found to correlate significantly with increasing FC, while an additional 24 genera were found to be associated with FC and/or SCCAI (16S-seq). Bacterial markers (DNA test) for Proteobacteria, *Shigella* spp. and *Escherichia* spp., were significantly correlated with increasing FC measures, while another 14 markers were found to be associated with FC and/or SCCAI.

Conclusions: In patients with UC, results of both methods are associated with disease activity, correlating significantly with *Peptostreptococcus anaerobius* (16S-seq) and with Proteobacteria, *Shigella* spp. and *Escherichia* spp. (DNA test).

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Introduction

Inflammatory bowel disease (IBD), including ulcerative colitis (UC) and Crohn's disease (CD), are chronic inflammatory diseases of the gastrointestinal tract that result from complex interactions of the intestinal immune system and the gut microbiota [1]. IBD is relapsing-remitting and approximately 30–50% of IBD patients experience flare-ups on a yearly basis [2–4]. Screening for IBD activity using electronic health (eHealth) applications has previously been shown to significantly reduce the time to remission relative to standard care (median 18 vs. 77 days) [5]. One plausible reason for eHealth solutions bringing about remission more quickly than standard care is that they involve patients in their own disease and its screening, especially in the form of home-monitoring, and that a relapse is thus registered sooner than with standard care [6]. Furthermore, some applications have incorporated a faecal calprotectin (FC) home point-of-care test

(POCT) in order to receive quick test results and thereby being able to initiate or change medical treatments sooner than using a conventional FC laboratory test [7–9]. FC has shown good predictive value for warning of relapses in UC [10,11]. Close monitoring of disease activity and quick initiation of treatment are vital for optimising individualised treatments in IBD and for potentially improving the long-term disease course [6,9,12]. Recently, a perturbed gut microbiome (dysbiosis) and its metabolic functions has been implicated as an essential factor in triggering inflammation in IBD, rather than its being a consequence of inflammation [1,13–15]. However, the bacterial dysbiosis in UC and CD is poorly understood in relation to clinically relevant parameters such as medications being taken, disease activity measures and disease course, and therefore the collecting of faecal samples for microbiota analysis has not yet been widely adopted for clinical use in optimising individualized treatments of IBD.

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 Supplemental data for this article can be accessed [here](#).

The primary aim of this cohort study was to explore possible associations between faecal microbiota (analysed in two different ways) and disease activity measures in adult IBD patients. The clinical perspective hereof was a possible future integration of a microbiota measure in an existing eHealth application for disease monitoring and quicker initiation or adjustment of treatment in IBD.

Materials and methods

We investigated associations between the faecal microbiota in adult IBD patients and various disease activity measures (faecal calprotectin (FC), Simple Clinical Colitis Activity Index (SCCAI), Harvey–Bradshaw Index (HBI), and total inflammation burden scoring (TIBS)), as well as medical therapies and phenotypes, in a cohort for one year.

Source population

In a previous study at the outpatient clinic at North Zealand University Hospital, Denmark, 120 adult IBD patients undergoing treatment at the time were randomised to home monitoring for disease activity for one year [16]. These patients fulfilled the Copenhagen diagnostic criteria for IBD [2,3] and were either in clinical remission (SCCAI ≤ 2 or HBI < 5), or had mild-to-moderate disease activity (SCCAI 3–4 and HBI 5–16) at the time of inclusion. In this IBD protocol patients were randomised to screen for disease activity according to the total inflammation burden scoring (TIBS) algorithm on the web application Constant Care[®], either every third month or whenever they felt a need for screening (i.e. on demand). The TIBS algorithm is a composite of a subjective score, either SCCAI [17] for UC or HBI [18] for CD, plus the result of a validated FC home testing kit [7]. FC measurements were performed by the patients at home using the CalproSmart[™] application (Calpro AS, Norway). This home

test can be performed in 18 min and is integrated into the Constant Care web application, providing the patients with an opportunity to see their SCCAI, HBI, FC and TIBS results longitudinally and in a traffic light form on the IBD Constant Care web application [6]. A one-year disease course in the Constant Care application and the cut-off values defining remission (green zone), mild-to-moderate activity (yellow zone), and severe activity (red zone) for the SCCAI, HBI, FC and TIBS are shown in Figure 1. Montreal classifications [19,20] (extent (UC), location and behaviour (CD)) were sourced from electronic patient files at inclusion, and the most severe stage of disease recorded was that used. Four patients were classified as IBD unclassified (IBDU); three of them were considered to be UC and one CD. Medical registrations were carried out retrospectively and based on both electronic patient files and the electronic prescription management system.

Study population

A sub-group of patients ($n = 78$) from the source population consented to provide repeated faecal samples while TIBS screening for disease activity on the Constant Care web application; the first and last patient consenting to participate in this study were enrolled 31 August 2015 and 25 July 2016, respectively.

Disease activity measures, phenotypes and medication were registered as described in the main protocol. Medications were registered in this cohort for all faecal samples and categorized as follows: None: no IBD medication; 5-aminosalicylic acid (5-ASA): oral mesalazine and sulfasalazine, including enemas and suppositories; Immunosuppressants: Azathioprine (and derivatives), methotrexate and steroids; Biologicals: any anti-TNF treatment, vedolizumab (anti- $\alpha 4\beta 7$ -integrin); and Combination therapy: any combination of the medications listed above. The Ethical Committee in Denmark

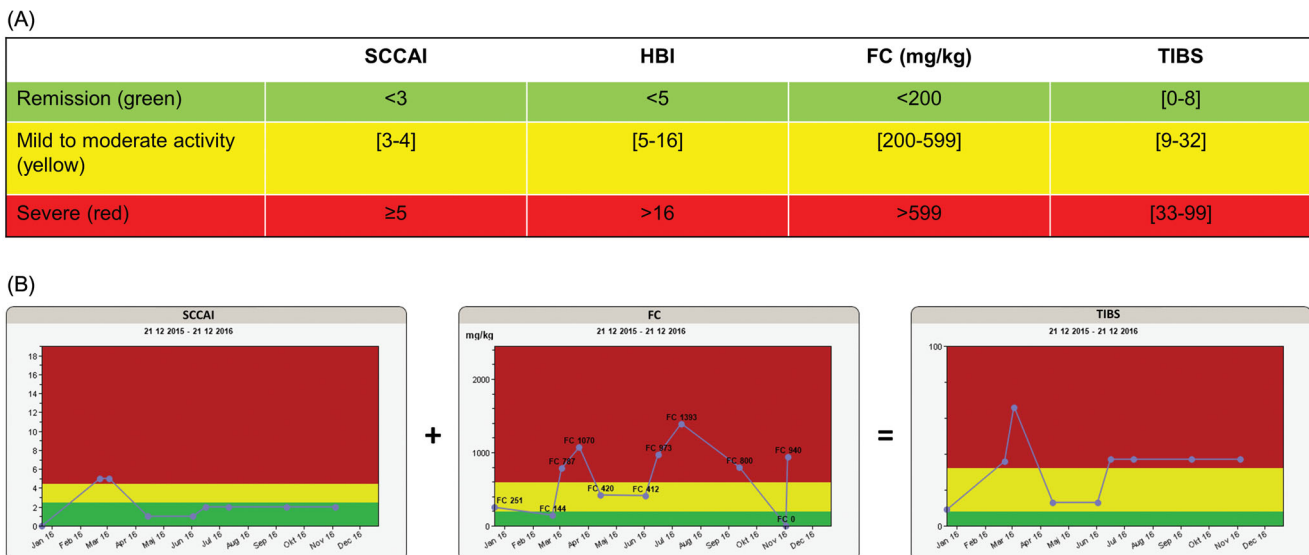


Figure 1. Disease algorithm in the web application Constant Care[®] for home monitoring of disease activity in patients with inflammatory bowel disease (ibd.-constant-care.com). (A) Cut-off values of disease activity scores (Simple Clinical Colitis Activity Index (SCCAI), Harvey-Bradshaw Index (HBI), faecal calprotectin (FC) home test, and total inflammation burden score (TIBS)) defining remission, mild-to-moderate activity, and severe activity. (B) An example of one-year of follow-up in Constant Care by a patient with ulcerative colitis.

approved the study (H-15005603) on July 22, 2015. The Danish Data Protection Agency approved the research biobank: I Suite no.: 03904, NOH-2015-028 and ClinicalTrials.gov ID: NCT02526251. All patients included in this cohort provided written, informed consent.

Microbiota analysis procedures

Patients who agreed to send in faecal samples for microbiota analysis were given easy sampler kits and faecal tubes and instructed to send in samples every time they used the home-monitoring system. All faecal samples were immediately frozen (-20°C) upon receipt at the hospital. All faecal samples were shipped (UN 3373) on dry ice (UN 1845) to Genetic Analysis AS, Norway for DNA extraction and microbiota analysis. A part of the extracted gDNA was shipped back to Denmark on dry ice for 16S-seq microbiota analysis at Statens Serum Institut, Copenhagen, Denmark.

The faecal microbiota (gDNA) were analysed in two different ways:

1. Commercially available GA-map[®] Dysbiosis Test, Genetic Analysis AS, Oslo, Norway (DNA test). The test is constructed of 54 bacterial markers and utilizes a pre-targeted hybridization approach based on amplification of the bacterial 16S rRNA gene of seven variable regions (V3-V9), utilising the Cover All amplicon developed by Genetic Analysis. Bacterial DNA labelling uses the single-nucleotide primer extension (SNUPE) technique and hybridization to complementary DNA strands coupled to beads. Signal detection was carried out using Bio-Code 1000A 128-Plex Analyzer (Applied BioCode, Santa Fe Springs, CA, USA). The results are provided as normalised signal for the 54 bacterial markers and as a dysbiosis index (DI; range 1–5), where a DI of 1–2 are considered as normobiosis, 3 mild dysbiosis and 4–5 severe dysbiosis. The DNA test has been described in detail elsewhere [21].
2. Microbiota analysis (based upon gDNA from Genetic Analysis) was also performed at Statens Serum Institut by sequencing the variable V3-V4 regions in the 16S rRNA gene, using primers described by Klindworth et al. [22], with the addition of Illumina overhang sequences suitable for Illumina dual indexing (16S-seq). PCR cycles, primers and sequence classification are described in Supplementary Comment C1.

Statistical analyses

Analysis of sequence counts and visualization of results was performed in R [23] version 3.5.0 using packages phyloseq [24], vegan [25], gplots [26], ggplot2 [27], plotly [28] and lme4 [29]. Sequence counts were rarefied to 11,754 using phyloseq (random seed set to 100), removing 61 of 710 taxa and one sample (no DNA). Only sequences that accounted for at least 0.05 percent of the sequences in one sample were included (16S-seq). Dissimilarity based analyses of 16S-seq data were performed based on Bray-Curtis dissimilarity

and visualized using Principal coordinate analysis (PCoA) while dissimilarity based analyses of DNA test data were based on Euclidean distances of log₂-transformed data and visualized using Principal component analysis (PCA). PCoA/PCA was performed on baseline samples and/or median sequence counts from each patient and was evaluated with analysis of similarities (ANOSIM) tests.

Alpha diversity was measured with the Shannon diversity index and number of observed species in 16S-seq and compared between groups using the Mann-Whitney U test. The dysbiosis index (DNA test) was used to compare differences between groups. Concordance between the dysbiosis index and severity measures was evaluated using the Kruskal-Wallis test while concordance between dysbiosis index and extent of UC and medication groups were compared with chi-square tests.

Univariable linear mixed effect models (LME) were fitted to predict log-transformed disease scores using normalised relative abundances of 125 genera (16S-seq) that were found in at least three samples, and 54 log₂-transformed normalised signals (DNA test) as fixed effects and identifiers for individual patients as random effects. A *p*-value for each genus was obtained by likelihood ratio tests comparing a null model with only patient identifier as random effect to a model with both genus/bacterial marker as fixed effect and patient identifier as random effect. The purpose of this was to evaluate whether genus/bacterial markers offered any additional explanation of the disease score that was not explained by differences between patients. All genera/bacterial markers with a *p*-value smaller than .05 are reported, together with adjusted *p*-value (Bonferroni correction for multiple testing) and *t*-values. Residuals from the null model were inspected with Q-Q plots and found to be close to the Gaussian distribution.

Results

Basic characteristics of the cohort

Seventy-eight IBD patients (65% of the original study population) provided a total of 288 faecal samples (UC: 234, CD: 32, and IBDU: 22) for microbiota analysis. Sixty-four (82%) provided more than one sample during the course of the one-year study period (median 3, range 2–11), while 14 patients (18%) provided only one sample. Two hundred and eighty-seven samples were successfully analysed by DNA test and 16S-seq, however, 15 samples were excluded post 16S sequencing (16S-seq) due to few reads leaving the total number of samples for 16S-seq to 272. Baseline characteristics of patients included in the cohort are shown in Table 1.

Bray-Curtis dissimilarity and Euclidian distance between samples from the same patient were, 16S-seq: median 0.430, IQR 0.350–0.530, DNA test: median 6.100, IQR 4.490–7.540. These distances were significantly lower than between samples from different patients 16S-seq: median 0.690, IQR 0.620–0.780, DNA test: median 9.820, IQR 8.630–10.980; Mann-Whitney U test, 16S-seq: *p* < .001, DNA test: *p* < .001. The magnitude of change in microbiota composition (Bray-Curtis dissimilarity/Euclidian distance) between consecutive samples was not significantly associated with the

Table 1. Baseline characteristics of patients with inflammatory bowel disease (IBD), ulcerative colitis (UC), Crohn's disease (CD) and IBD unclassified (IBDU).

	IBD	UC	CD	IBDU
Total	78 (100)	63 (80.8)	11 (14.1)	4 (5.1)
Sex				
Male	41 (52.6)	32 (50.8)	7 (63.6)	2 (50.0)
Female	37 (47.4)	31 (49.2)	4 (36.4)	2 (50.0)
Mean body mass index (SD; kg/m ²)	25.7 (4.7)	25.8 (4.9)	26.3 (4.3)	23.2 (2.3)
Smoking				
Current	6 (7.7)	4 (6.3)	1 (9.1)	1 (25.0)
Former	44 (56.4)	37 (58.7)	6 (54.5)	1 (25.0)
Never	28 (35.9)	22 (34.9)	4 (36.4)	2 (50.0)
Mean age at inclusion (SD; years)	48.7 (14.7)	48.4 (13.8)	51.5 (17.9)	46.0 (22.3)
Mean age at diagnosis (SD; years)	36.8 (13.7)	37.0 (12.5)	34.8 (18.2)	39.5 (21.4)
UC extent				
E1, proctitis		21 (33.3)		
E2, left-sided		25 (39.7)		
E3, extensive		17 (27.0)		
CD location				
L1, small bowel			1 (9.1)	
L2, colonic			1 (9.1)	
L3, ilea-colonic			9 (81.8)	
CD behaviour				
B1, inflammatory			8 (72.7)	
B2, stricturing			3 (27.3)	
B3, penetrating			0	
Previous abdominal surgery	6 (7.7)	0	6 (54.5)	0
Disease duration (IQR; years)	8.5 (4.0-16.5)	7.0 (4.0-16.0)	15 (6.0-29.0)	6.5 (1.8-12.8)
Medical treatment				
None	11 (14.1)	5 (7.9)	5 (45.5)	1 (25.0)
5-ASA	45 (57.7)	42 (66.7)	0	3 (75.0)
Immunosuppressants	3 (3.8)	1 (1.6)	2 (18.2)	0
Biological	3 (3.8)	2 (3.2)	1 (9.1)	0
Combination therapy	16 (20.5)	13 (20.6)	3 (27.3)	0
Education ^a				
Short	6 (7.7)	6 (9.5)	0	0
Student	4 (5.1)	3 (4.8)	0	1 (25.0)
Medium	54 (69.2)	42 (66.7)	9 (81.8)	3 (75.0)
Higher/Academic	14 (17.9)	12 (19.0)	2 (18.2)	0
Employed				
Yes	64 (82.1)	53 (84.1)	8 (72.7)	3 (75.0)
No	14 (17.9)	10 (15.9)	3 (27.3)	1 (25.0)
Patient-reported co-morbidities				
None	33 (42.3)	31 (49.2)	2 (18.2)	0
1	32 (41.0)	20 (31.7)	9 (81.8)	3 (75.0)
More than 1	11 (14.1)	11 (17.5)	0	0
Missing	2 (2.6)	1 (1.6)	0	1 (25.0)

Data are expressed as number (%), median (IQR) or mean (SD).

^aMedium length education defining people with a 3–4 years education after high school, e.g. nurses, technical people and bachelors' degrees. Higher/Academic defined as a master's degree or higher.

time (number of days) between sampling (Spearman's correlation: $\rho = 0.200$, $p = .120$ (16S-seq) and $\rho = 0.090$, $p = .480$ (DNA test), [Supplementary Figure S1](#)).

Based on the median figures of all faecal samples for each patient, there was a greater median Shannon diversity in patients with UC than in patients with CD ($p = .030$, [Figure 2\(A\)](#)). In addition, the diagnoses of CD and UC were significantly separated by both microbiota methods; ANOSIM, $R = 0.400$, $p = .002$ (16S-seq) and $R = 0.320$, $p = .001$ (DNA test), [Figure 2\(B,D\)](#). There was no significant difference ($p = .190$) in DI scores between the diagnoses when grouped into normobiosis (DI 1-2), mild dysbiosis (DI 3) and severe dysbiosis (4-5), [Figure 2\(C\)](#).

Shannon diversities and DI scores based on baseline data and grouped by extent (E1, E2, E3) in UC patients are shown in [Figure 3\(A,B\)](#). Pairwise comparisons of baseline Shannon diversity between the phenotypes of UC E1 (proctitis), E2 (left-sided) and E3 (extensive) showed significantly higher alpha-diversity in E1 compared to E2 ($p = .002$) and E3

($p = .010$). No significant association between DI scores and the extent of UC was found ($p = .910$), [Figure 3\(B\)](#). Neither 16S-seq nor the DNA test were able to distinguish between UC phenotypes based on ANOSIM tests (16S-seq: $R = 0.016$, $p = .280$ and DNA test: $R = 0.019$, $p = .240$). Assuming that the patients' disease did not progress during the observation period, median operational taxonomic unit (OTU) counts (16S-seq) and median normalised signal (DNA test) for each UC patient showed the same pattern as at baseline (data not shown). Too few CD samples were collected from this cohort to evaluate potential clustering by location and behaviour, [Supplementary Figure S2](#). Due to the relatively small number of CD faecal samples ($n = 36$), further analyses were primarily based on UC disease data and microbiota.

There was no statistical difference in Shannon diversity, DI scores and microbiota composition in relation to medications taken for UC patients whose medication did not change during the study period, [Supplementary Figure S3](#), nor for UC patients whose medication changed (data not shown).

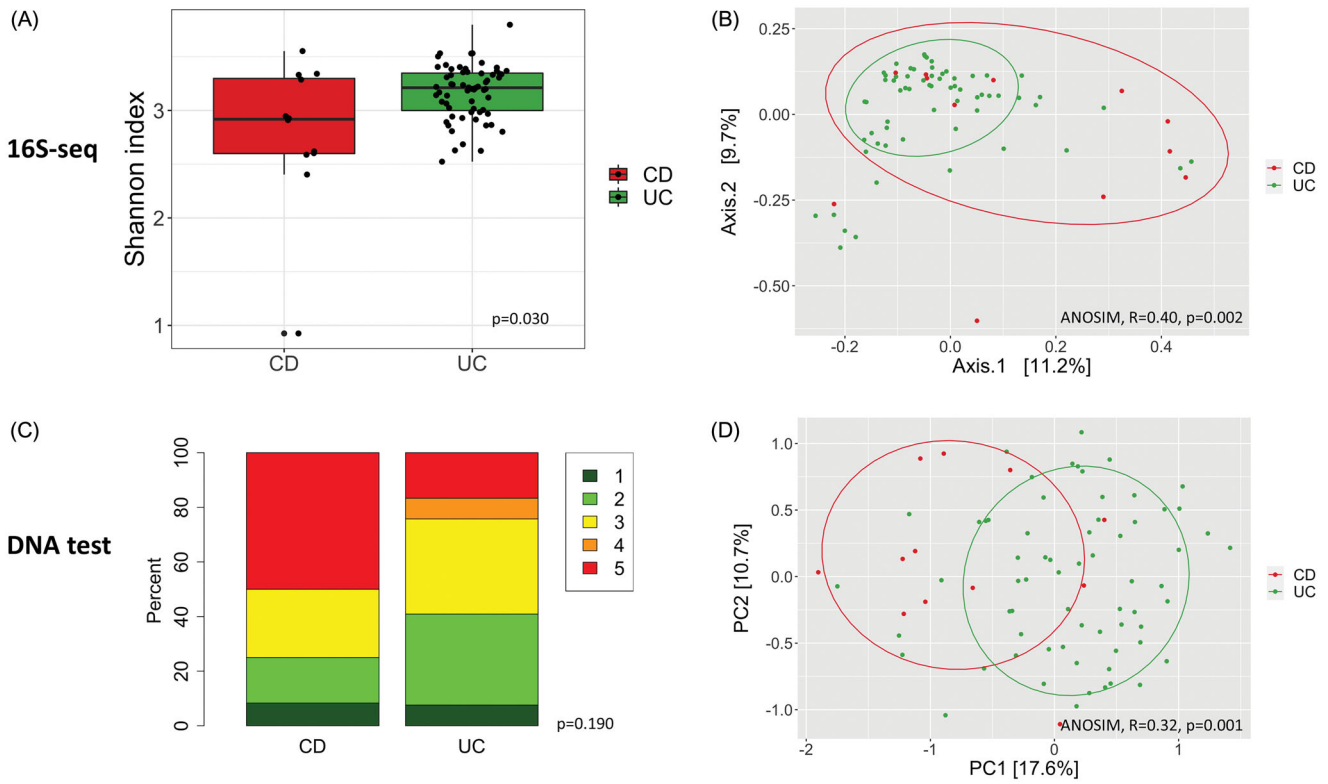


Figure 2. Microbiota profiles differ in Crohn's disease (CD) and ulcerative colitis (UC). All figures are based on medians for CD ($n = 12$) and UC ($n = 64$). (A) Alpha diversity (Shannon index) by CD and UC diagnoses based on 16S-seq. (B) Principal coordinate analysis (PCoA) by CD and UC diagnoses based on 16S-seq. (C) Dysbiosis index (1–2: normobiosis, 3: mild dysbiosis, and 4–5: severe dysbiosis) by diagnosis CD and UC and Kruskal–Wallis, $p=.190$. (D) Principal component analysis (PCA) by CD and UC diagnoses based on DNA test. Ellipses (B and D) are drawn around UC and CD samples corresponding to a 75% confidence level, indicating a 75% chance that a sample from an UC or CD patient occur within this range.

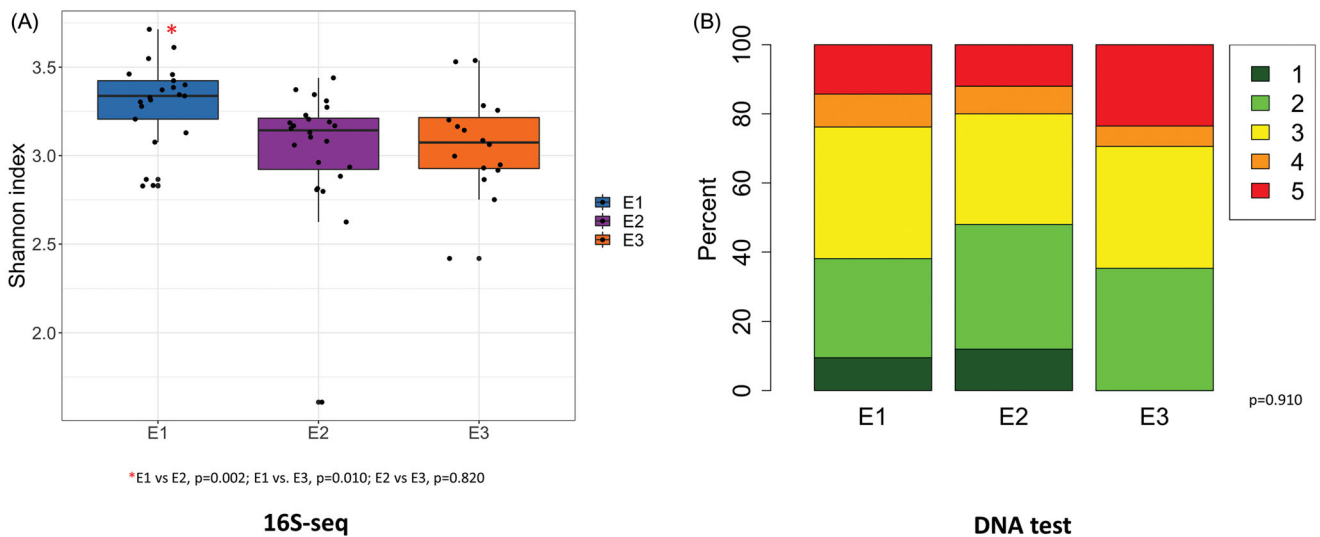


Figure 3. Microbiota and Montreal classification in ulcerative colitis (UC) as determined by 16S-seq and DNA test. (A) Alfa diversity (Shannon index) by disease extent (E1, E2, E3) at baseline ($n = 61$; 61 samples) showing a decreased diversity in left-sided disease (E2, $n = 24$) and pancolitis (E3, $n = 16$) relative to proctitis (E1, $n = 21$). (B) Dysbiosis index: 1–2: normobiosis, 3: mild dysbiosis, and 4–5: severe dysbiosis (DNA test) by extent at baseline ($n = 61$; 61 samples) and chi-square, $p = .910$. The disease extent given is the most severe stage recorded for each UC patient.

Microbiota and disease activity

The 10 most abundant bacterial genera/bacterial markers in UC and CD in relation to disease activity measures of FC, HBI, SCCAI, TIBS, Montreal classification, medication and DI are shown in [Supplementary Figure S4\(UC\)](#) and [S6\(CD\)](#) for 16S-seq and [Figure S5\(UC\)](#) and [S7\(CD\)](#) for DNA test. Samples

are ordered according to a hierarchical clustering based on all genera/bacterial markers. For UC patients ([Supplementary Figure S4](#)) four groups were identified in which a high degree of patient clustering was observed. Two of the groups had mixed disease activity (remission or activity) and had either relatively high abundance of *Bacteroides* or *Blautia*. The last two groups showed either to be in remission

(primarily) and having relatively high abundance of *Prevotella* or having moderate to severe disease activity and relatively high abundance of *Escherichia*. DNA test for UC patients (Supplementary Figure S5) revealed only two obvious groups; one with many severe disease activity measures, high DI scores and reduced abundance of the 10 most abundant bacterial markers and the other group showing mixed disease activity.

The DI approach (normobiosis (DI 1–2), mild dysbiosis (DI 3) and severe dysbiosis (4–5)) was illustrated at baseline for all UC patients according to disease severity measures (Figure 4), with SCCAI and TIBS showing significant associations ($p < .02$).

Bray–Curtis dissimilarity (16S-seq) and Euclidian distances of log₂-transformed data (DNA test) between baseline samples, grouped by disease severity (FC, SCCAI and TIBS), are shown in Supplementary Figure S8. The Bray–Curtis dissimilarity was found to be significantly smaller between patients within the mild FC category (green, mild-mild) than between patients within the severe FC category (red, severe-severe) ($p = .012$, 16S-seq). Similarly, when comparing samples from the mild FC group to samples from the severe FC group (mild-severe) the Bray–Curtis dissimilarity was significantly greater than the dissimilarity between samples within the mild group (green, mild-mild) ($p < .001$, 16S-seq). The same trend was not observed when looking at SCCAI severity for 16S-seq. DNA test data showed highly significant differences: mild-mild to severe-severe and mild-mild to mild-severe were $p < .001$ for all disease severity scores (FC, SCCAI and TIBS), Supplementary Figure S8.

Linear mixed-effects models (LME) for 16S-seq data using identifiers for individual UC patients as random effect identified one genus, *Peptostreptococcus*, which was significantly correlated with increased FC after Bonferroni correction, none for SCCAI, and one (*Peptostreptococcus*) with increased TIBS. *Peptostreptococcus* – of which approximately 80% could be classified on the species level as *P. anaerobius* – was mostly found in low abundances and represented between 0 and 0.91% of the sequence found in any one sample. Additionally, 24 genera were found to be associated (although not statistically significant after Bonferroni correction) with FC and/or SCCAI, see heatmap Figure 5(A) (and Supplementary Table S1).

LME based on DNA test data found Proteobacteria, *Shigella* spp. and *Escherichia* spp., to be correlated with increased FC. An additional 14 bacterial markers were found to be associated with an increase or decrease in one or more of the severity measures, see heatmap Figure 5(B) (and Supplementary Table S1).

Discussion

We have demonstrated that two methods of analysing microbiota, DNA test and 16S-seq, are able to characterise the microbiota of IBD patients. Both methods were able to differentiate between diagnoses of UC and CD, and 16S-seq was also capable of identifying less diverse microbiota when comparing CD to UC and left-sided/extensive disease to

proctitis. In addition, greater Bray–Curtis and Euclidian variability were observed among UC patients with severe disease activity relative to patients in remission. Increasing FC levels in UC was correlated with *Peptostreptococcus anaerobius* when using 16S-seq. Proteobacteria and *Shigella* spp. and *Escherichia* spp. were positively correlated with increasing FC in UC patients when using the DNA test.

It has become evident in recent years that the ecosystem of the gastrointestinal tract, including the microbiota, plays a vital role in human health and disease. In study after study, health and disease have been associated with the microbiota, including IBD, obesity, atherosclerosis, multiple sclerosis, psoriasis, rheumatoid arthritis, diabetes, psychiatric and neurodegenerative diseases [30]. The results of this study offer further evidence that the faecal microbiota are linked to basic disease characteristics in adult IBD patients: CD patients had significantly less diverse microbiota than UC patients, and the microbiota of the majority of CD patients were distinct from UC patients. Similar results have been described by other researchers [31]. Furthermore, UC patients with proctitis had a significantly greater Shannon diversity relative to UC patients that had progressive disease; however, we failed with both DNA test and 16S-seq to separate UC phenotypes based on ANOSIM-test. Similarly, Franzosa et al. [15], who performed a metagenomic and metabolomic study on IBD patients, were not able to separate 68 CD patients based on phenotypes; however, the authors argued that a plausible reason for not being able to separate the CD patients according to disease progression was the established nature of IBD within their PRISM cohort. Phenotyping of CD and UC patients in this cohort was not based on endoscopy at inclusion in the study, but rather the most severe stage of progression noted in their medical records.

Interestingly, few studies have been published on IBD medical therapies and how they affect the microbiota, along with any correlations to disease activity or progression in disease. However, Halfvarson et al. [32] have demonstrated that a change in medication influences the volatility of the microbiome. Moreover, Ananthakrishnan et al. demonstrated that a more diverse microbial composition and 13 functional pathways at baseline could predict remission after week 14 in patients with CD undergoing treatment with vedolizumab [33], and Morgan et al. [34] found that age, smoking, sample biogeography, and IBD medical treatment were strongly associated with microbiota composition. In the latter study, treatment with 5-ASA was correlated with a large reduction in *Escherichia/Shigella*, immunosuppressants and 5-ASA were associated with moderate increases in *Enterococcus*, and antibiotic treatments were strongly associated with a reduction in biodiversity. The present data on microbiota and IBD medication did not lead to anything conclusive.

In UC in particular, FC has been proven as a non-invasive, clinically valuable marker for monitoring disease activity [35–37] and has just recently been shown to correlate to metabolic activity in IBD; the same study also found a similar significant correlation when looking exclusively at UC patients ($n = 25$, $r = 0.565$, $p = .003$) [15]. In our study, increasing FC severity correlated most strongly with an

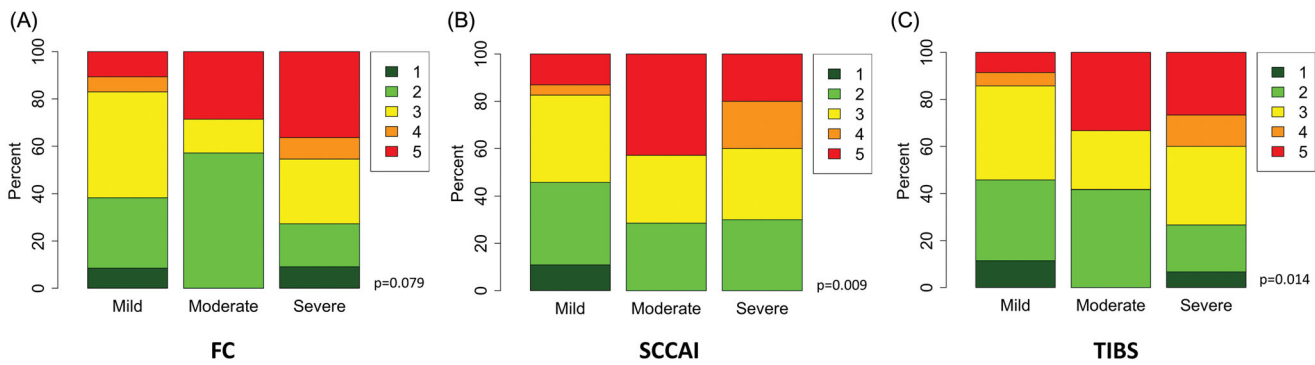


Figure 4. Stacked bar plots of DNA test and dysbiosis index (DI) in relation to disease severity at baseline in patients with ulcerative colitis (UC), $n = 64$. (A) DI (1–2: normobiosis, 3: mild dysbiosis, and 4–5: severe dysbiosis) in relation to faecal calprotectin (FC) and Kruskal–Wallis, $p = .079$. (B) DI in relation to Simple Clinical Colitis Activity Index (SCCAI) and Kruskal–Wallis, $p = .009$. (C) DI in relation to total inflammation burden score (TIBS) and Kruskal–Wallis, $p = .014$.

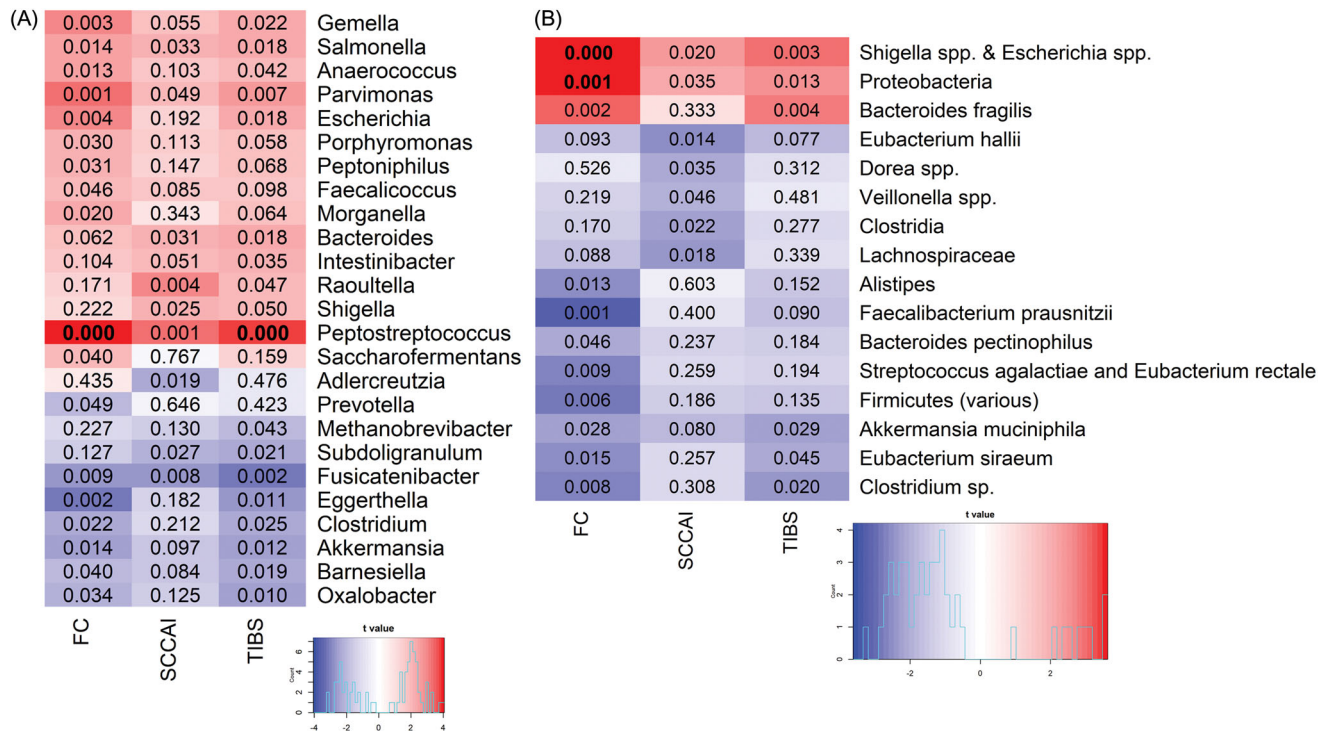


Figure 5. Heatmaps illustrating associations between disease activity scores; faecal calprotectin (FC), Simple Clinical Colitis Activity Index (SCCAI), total inflammation burden score (TIBS) and (A): genera based on 16S-seq and (B): bacterial probes (DNA test). Associations are calculated based on linear mixed-effects models (LME) using individual identifiers for each patient as random effects ($n = 64$, 237 samples) and specific bacterial genera/probes as fixed effects. The t value is the correlation estimate based on LME. Red indicates positive correlation with increased severity, blue indicates negative correlation. The stronger the colour the greater the association. P -values listed in cells are uncorrected p -values and Bonferroni corrected p -values are highlighted in bold. The heatmaps include all genera/markers with significant ($p < 0.05$) associations before multiple testing correction. For more detailed information of t -values for specific genera and bacterial probes see [Supplementary Table S1](#). *Firmicutes/Tenericutes/Bacteroidetes species.

abundance of *Peptostreptococcus anaerobius*, as determined by 16S-seq. *Peptostreptococcus* species are members of the normal microbiota, e.g. in the mouth and gastrointestinal tract, and is one of the most common Gram-positive anaerobic cocci associated with infections of the abdominal cavity [38]. They can, however, often be found in poly-microbial infections. Most poly-microbial infections in the gastrointestinal tract, including *Peptostreptococcus*, originates from mucus bacteria adjacent to the infected site where *Bacteroides fragilis* and Enterobacteriaceae predominate [39]. In the present study, *B. fragilis* was associated, in UC, with the inflammatory stool marker FC. Furthermore, members of the Enterobacteriaceae family, including Proteobacteria, *Escherichia*

spp. and *Shigella* spp., which are bacterial markers of the DNA test, correlated significantly with FC among UC patients. The hierarchical clustering of samples showed a relatively high degree of patient clustering and clusters of patients with high levels of *Escherichia* spp. (16S-seq); however, *Escherichia* did not correlate significantly with disease activity (16S-seq) after Bonferroni correction, and nor did a previous meta-analysis of IBD and disease activity [40] document such a relationship between these bacterial species, which could indicate that *Escherichia* spp might not be a true marker of disease activity alone but rather as a part of the disease steady state/dysbiosis and IBD chronicity, as suggested by Morgan et al. [34]. Firmicutes, the butyrate-producing and ‘peace keeping’

Faecalibacterium prausnitzii, showed a weak negative correlation to FC when using the DNA test method. Machiels et al. [41] have shown that *Faecalibacterium prausnitzii* are less abundant in the dysbiosis of UC patients. *P. anaerobius* have, to our knowledge, not been shown to correlate significantly with disease activity in UC before now. However, they have previously been associated with colorectal cancer (CRC) [42] and Dejea et al. [43] just recently showed that two bacteria, *B. fragilis* and a strain of *E. coli*, collaborated in generating biofilms and together exacerbate the growth of tumours.

Longstanding disease, as well as high inflammation burden and disease progression, have also been linked to increased risk of CRC in UC patients and therefore surveillance programs have been established in most European countries. If these associations are accurate, disease activity (and maybe disease progression) and CRC might also be related *via* poly-microbial biofilms including *Peptostreptococcus*; however, further studies are needed to verify these hypotheses. It should also be noted in this context that FC is not a specific marker for disease activity in UC, for FC can also be elevated in cases of infective gastroenteritis and CRC, among other diseases [37].

The dysbiosis index did help establish a relationship between all disease activity scores in UC (FC, SCCAI and TIBS) and baseline samples in this study; however, due to uneven numbers of samples for each patient it has not been possible to test statistically these relationships over time. Lloyd-Price et al. [44] found no significant relationship between FC and dysbiosis score (0-1) in their multi-omics study of IBD; but they did document how periods of disease activity in IBD were marked by increases in temporal variability, with characteristic taxonomic, functional and biochemical shifts. We found greater Bray–Curtis and Euclidian variability among UC patients with severe disease activity relative to patients in remission. This effect was more clearly detected by the DNA test method than 16S-seq on all disease activity measures.

Data from this study on bacterial diversity, variability, chronicity and correlations with disease activity measures substantiate, in part, previous descriptions of UC as a polymicrobial infectious disease of the colon that is characterised by a sustained broken mucus barrier, bacterial migration toward the mucosa and complex bacterial biofilms on the epithelial surface [45]. This would encourage (1) future research into new medical treatments of UC that target bacterial diversity, polymicrobial infections and biofilms, and (2) integrative disease monitoring, together with other clinically relevant measures (e.g. SCCAI and FC), of the bacterial dysbiosis and chronicity in clinical (web) practice.

The strength of this study is that faecal microbiota are analysed in two different ways and correlated by LME to a variety of disease parameters across multiple patients over the course of one year. However, our analysis is hindered by a large discrepancy in the number of samples obtained from each patient. Another limitation is that this cohort is a single centre study and that the study design does not allow for extraction of causal relationships between the microbiota, IBD medications being given and a patient's disease progression.

In summary, these results indicate that the faecal gut microbiota in IBD are associated with disease activity. *Peptostreptococcus anaerobius* (as determined by 16S-seq), and *Shigella* spp. and *Escherichia* spp. (as determined by DNA test) were correlated with increasing FC in patients with UC. Moreover, UC patients with proctitis as their most severe stage of disease progression had a greater Shannon diversity than those with left-sided and extensive disease. Before implementing any of the two microbiota methods in clinical (web) practice, further larger and longitudinal studies are needed, validating the two tests in relation to the TIBS disease activity algorithm as compared against the golden standard, which remains endoscopy.

Disclosure statement

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Author contributions

DVA prepared the manuscript, which was critically reviewed by all co-authors. DVA, DM, JB and PM designed the study. DM, PM, DVA conducted the study. DVA and TJ had full access to study data and take full responsibility for their integrity. TJ, ABK and BL carried out the bioinformatic analyses. All authors approved the final version of the manuscript. PM is the guarantor of the article.

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