

Mucosal 5-aminosalicylic acid concentration, drug formulation and mucosal microbiome in patients with quiescent ulcerative colitis

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Summary

Background: 5-aminosalicylic acid (5-ASA) is the first-line therapy for ulcerative colitis (UC). 5-ASA acts locally in the colonic mucosa by numerous proposed mechanisms, and is metabolised by N-acetyltransferase (NAT). Large variations in mucosal 5-ASA concentrations have been reported, but the underlying mechanisms are not understood.

Aim: To study the relationship between 5-ASA concentration, 5-ASA formulation, NAT genotype and bacterial microbiome in patients with UC.

Methods: Patients with quiescent UC, using monotherapy of Mezavant (n = 18), Asacol (n = 14) or Pentasa (n = 10), 4.0–4.8 g/day were included. 5-ASA was measured in colonic mucosal biopsies and serum by ultra-high performance liquid chromatography. NAT genotypes were determined by Sanger sequencing. Bacterial microbiome was sequenced from faeces and mucosa by 16S rRNA sequencing using Illumina Miseq.

Results: Mezavant provided the highest mucosal 5-ASA levels (geometric mean 2.39 ng/mg), followed by Asacol (1.60 ng/mg, 33% lower, $P = 0.50$) and Pentasa (0.57 ng/mg, 76% lower, $P = 0.033$). Mucosal 5-ASA concentration was not associated with NAT genotype, but serum 5-ASA concentration and NAT1 genotype was associated ($P = 0.044$). Mucosal 5-ASA concentration was positively associated with mucosal bacterial diversity ($P = 0.0005$) and bacterial composition. High mucosal 5-ASA concentration was related to reduced abundance of pathogenic bacteria such as Proteobacteria, and increased abundance of several favourable bacteria such as Faecalibacterium.

Conclusions: Mucosal 5-ASA concentration is positively associated with bacterial diversity and a mucosal bacterial composition that are perceived favourable in UC. Mezavant yielded higher mucosal 5-ASA concentrations than Pentasa. 5-ASA may have beneficial effects on the mucosal microbiome, and high concentrations possibly amend dysbiosis in UC.

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1 | INTRODUCTION

Mesalazine or 5-aminosalicylic acid (5-ASA) is the first line therapy for patients with ulcerative colitis (UC), and is effective both for inducing and maintaining remission.^{1,2} 5-ASA is proposed to act through numerous mechanisms, including inhibition of pro-inflammatory mediators such as leukotriens, prostaglandin, interleukin-1, Nuclear factor- κ B (NF- κ B) and tumour necrosis factor alpha (TNF α), as well as a peroxisome proliferator-activated receptor gamma (PPAR- γ) receptor agonist (reviewed by Lichtenstein and Kamm³).

5-ASA exerts its effect in the intestinal mucosa, and mucosal 5-ASA concentrations are inversely correlated to disease activity.⁴⁻⁷ It is therefore important to identify and understand the determinants of mucosal 5-ASA concentration. In the intestinal mucosa, 5-ASA is acetylated to its inactive metabolite N-acetyl-5-ASA (Ac-5-ASA), mainly by the enzyme N-acetyl-transferase 1 (NAT1), and to a small degree by N-acetyl-transferase 2 (NAT2).^{8,9} Orally administered unbound 5-ASA is absorbed and inactivated in the small intestinal mucosa and in the liver, thus only small amounts 5-ASA will reach the colonic mucosa.^{3,10} Therefore, several pharmaceutical delivery systems have been developed to transport orally administered 5-ASA to the colon. In a time-dependent formulation (Pentasa, Ferring Pharmaceuticals, Saint-Prex, Switzerland), 5-ASA is coated with a semipermeable membrane of ethyl cellulose, providing a pH-independent release of approximately 35% of its content in the small bowel, 25% in the colon, while the last 40% eliminates in the faeces.¹¹ In a pH-dependent delivery system (Asacol, Tillots Pharma AG, Rheinfelden, Switzerland), 5-ASA is coated with Eudragit S which dissolves at a pH >7,¹² normally occurring in the terminal ileum and caecum. The Multi Matrix System (Mezavant, Shire Pharmaceutical Contracts Ltd, in partnership with Cosmo SpA, Milan, Italy) consists of hydrophilic and lipophilic excipients, covered by a pH-dependent coating dissolving at pH 7, causing slow diffusion of the drug into the gut lumen.³ For both Asacol and Mezavant absorption in the ileum is estimated to be around 20%.^{13,14} Faecal elimination for Asacol and Pentasa has been found to be similar.¹²

After oral administration of 5-ASA, mucosal concentrations are highest in the proximal colon segments and lowest in the rectum for the previously examined preparations.^{7,15,16} Higher concentrations in the rectum and left hemicolon have been achieved by combining oral and rectal 5-ASA formulations.^{16,17} However, rectal administration may be inconvenient and adherence to treatment over time is low. As 65%-85% of patients with UC have rectosigmoid and left-sided involvement at the time of diagnosis,^{18,19} it seems essential to assure optimal concentrations of 5-ASA in these bowel segments. Although previous studies suggest that Asacol yields higher mucosal 5-ASA concentrations than Pentasa,^{4,15} the different 5-ASA formulations are by many considered clinically equally efficient.² Studies comparing mucosal 5-ASA concentrations of the more recently marketed Mezavant with other 5-ASA formulations, have to the best of our knowledge, not been published.

Patients with UC have an altered gut microbiome, commonly called dysbiosis. Microbiome alterations in UC are characterised by reduced alpha-diversity²⁰⁻²³ combined with increases in the Proteobacteria phylum^{22,24,25} and decreases in the Firmicutes phylum.^{21,25,26} At genus level, Roseburia is reported to be decreased and Haemophilus increased.²⁷ The effect of selected antibiotics, probiotics and faecal microbiota transplantation²⁸ supports that dysbiosis is a part of the pathogenesis in UC. 5-ASA has previously also been reported to affect intestinal bacteria, by inhibiting growth of anaerobic strains, reducing bacterial invasiveness and total faecal bacterial abundance,²⁹⁻³¹ as well as reducing bacterial adherent biofilm thickness and Escherichia and Shigella abundances in patients with inflammatory bowel disease (IBD).^{21,32} Results from a recent study also suggest that 5-ASA acts as a polyP-kinase inhibitor, thereby decreasing some bacteria's ability to colonise and increase their susceptibility to oxidative stress.³³

In the current study, we aimed to measure and compare mucosal 5-ASA concentrations in the left hemicolon and rectum in patients with quiescent UC using different oral 5-ASA formulations (Mezavant, Asacol or Pentasa). We also aimed to explore underlying mechanisms which could explain variations in mucosal 5-ASA concentration as well as exploring the interrelation between 5-ASA mucosal concentration and bacterial composition.

2 | MATERIALS AND METHODS

2.1 | Study population

Patients were recruited from the Department of Gastroenterology and Hepatology, St. Olav's Hospital, Trondheim, Norway from 2015 to 2017. Inclusion criteria were confirmed diagnosis of UC in clinical remission prior to invitation, 18-70 years of age and use of oral 5-ASA (Asacol, Pentasa or Mezavant) 4.0-4.8 g once daily. Exclusion criteria were use of rectal 5-ASA formulations, prednisolone, azathioprine, methotrexate and TNF- α medication within the last 3 months. None of the patients had been using antibiotics or probiotics during the last 3 months prior to inclusion. The study was approved by the Regional Committee for Medical and Health Research Ethics, Central Norway (approval reference 2014/2043).

2.2 | Blood samples, endoscopic procedure and collection of mucosal biopsies

Eight hours after daily 5-ASA dose ingestion, patients underwent blood sample collection, enema bowel preparation and sigmoidoscopy. Blood haemoglobin (Hb), leucocyte concentration, plasma erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP) were analysed successively. Serum was stored at -80°C until analysis of 5-ASA and Ac-5-ASA concentrations. EDTA blood was used for NAT1 and NAT2 genotyping using Sanger sequencing, as described in Supporting Information. A 240 mL sorbitol enema (Klyx,

Ferring AS, Copenhagen, Denmark) was administered 30–45 minutes before sigmoidoscopy (Olympus Exera II GIF H180, Olympus Europa GmbH, Hamburg, Germany) up to 40 cm from the anal verge. Sets of three mucosal biopsies were collected at 10, 25 and 40 cm from the anal verge (total of nine biopsies) using endoscopy forceps (EndoJaw, Olympus Medical Systems, Tokyo, Japan) after cleaning of the mucosa with water to remove visible faecal remnants. Three biopsies were put on formalin for subsequent histological scoring of inflammation. The remaining six biopsies were briefly rinsed in NaCl 9 mg/mL solution, dried, weighed, snap frozen on liquid N₂, and stored at –80°C for subsequent measurement of 5-ASA and Ac-5-ASA concentration and sequencing of the mucosal microbiome.

2.3 | Analysis of 5-ASA and Ac-5-ASA concentrations

5-ASA and Ac-5-ASA concentrations were analysed in five mucosal colonic biopsies (two biopsies from 10 cm, one biopsy from 25 cm and two biopsies from 40 cm from the anal verge) and in serum from each patient, using an ultra-high performance liquid chromatography tandem mass spectrometry (UPLC-MS/MS) method, as described in Supporting Information.

2.4 | Evaluation of disease characteristics and inflammation

Demographic and clinical characteristics were recorded. Disease activity was assessed by clinical and endoscopic Mayo score,³⁴ Geboes histological score³⁵ and faecal calprotectin. Endoscopic remission was defined as an endoscopic Mayo score ≤ 1 , histological remission as a Geboes score < 2.1 and remission according to total Mayo score as a total Mayo score ≤ 2 . Patients that fulfilled both endoscopic Mayo score ≤ 1 and Geboes score < 2.1 were classified as being in deep remission.³⁶ Patients were instructed to bring a fresh (< 24 hours) faecal sample to the appointment. An aliquot was used for calprotectin measurement using a commercial ELISA method (Calpro AS, Lysaker, Norway), normal range < 50 mg/kg. Three biopsies from each patient, one from each location (10, 25 and 40 cm from the anal verge) were histologically evaluated by an experienced pathologist. The pathology assessment of inflammation was blinded and scored according to Geboes histological score.³⁵

2.5 | Microbiome analysis

The bacterial microbiome was analysed in one mucosal biopsy (sampled 25 cm from the anal verge) and in one faecal sample from each patient (stored at –80°C until analysis). Bacterial DNA was isolated using QIAamp Powerfecal (faecal samples) (Qiagen, Hilden, Germany) and DNeasy Powersoil kit (biopsy samples) (Qiagen), further described in Supporting Information. Libraries were constructed according to Illumina's 16S Metagenomic Sequencing Library Preparation. 16S rRNA amplicon sequencing was performed on an Illumina MiSeq sequencer (Illumina Inc, San Diego, CA). The short read

sequencing data from both faecal and mucosal origin were analysed using the operational taxonomic unit (OTU) approach. The QIIME software pipeline (version 1.9.1)³⁷ was used to cluster the gene sequences into OTUs based on sequence similarity. The SortMeRNA_SUMACLUSt method of open-reference OTU picking was applied to a total of 6207583 and 296047 sequencing reads and clustered into 1043 and 770 OTUs for faecal and mucosal samples respectively, at 0.8 SortMeRNA coverage threshold. The OTU taxonomy was identified using the Ribosomal Database Project classifier trained on the Greengenes database (version 13.8).

2.6 | Statistical analysis

IBM SPSS Statistics version 24.0 (IBM Corp., Armonk, NY) was used to conduct the statistical analysis. Demographic and clinical characteristics are presented as median (IQR, interquartile range) for skewly distributed variables, mean value (SD) for normal distributed variables, and as % (n) for categorical variables. Accordingly, Kruskal-Wallis test, *F*-test (ANOVA) and chi-squared test were used for comparing 5-ASA formulations groups with respect to these measures. A multilevel linear mixed model was applied to test for difference in mean mucosal concentrations of 5-ASA and Ac-5-ASA (log-transformed data) by type of 5-ASA formulation, adjusted for (no-interaction model), or specific to (interaction model) sample locations in the left hemicolon and rectum (10, 25 and 40 cm from the anal verge), further described in Supporting Information. Kruskal-Wallis test was used for comparing serum concentrations between the three 5-ASA formulations. Regardless of 5-ASA formulation ($n = 42$), mean mucosal 5-ASA concentration (individual level defined by mean of five repeated measures, log-transformed) and mean serum 5-ASA concentration was compared according to NAT genotype (two phenotypes for NAT1 and three phenotypes for NAT2) and disease activity (deep remission or remission according to endoscopic or total Mayo score) using one-way ANOVA. *P*-values < 0.05 were considered statistically significant. A power analysis was performed in which mean mucosal 5-ASA concentration estimates were based on previous studies comparing Pentasa and Asacol^{4,7,15} by one-way ANOVA and two pair-wise comparisons. With mean 5-ASA concentrations of 60 and 20 ng/mg, a SD of 30 ng/mg, $\alpha = 0.05$ and $\beta = 0.8$, 11 patients in each group were needed.

Associations between 5-ASA mucosal concentrations and bacterial composition in the mucosa and faeces were examined using a linear regression model. The OTU tables generated by the QIIME pipeline were imported into the R software environment version 3.4.1 (R Foundation for Statistical Computing, Vienna, Austria) using the phyloseq package and subsequently filtered as to include only OTUs from the Bacteria kingdom and excluding OTUs classified as Mitochondria, Chloroplast or Cyanobacteria/Chloroplast. Alpha diversity values were estimated from the filtered datasets using Shannon entropy. The core microbiome was estimated by requiring 10% prevalence of detected (> 1 sequence) OTUs. The core microbiome had a total of 822 and 156 OTUs in faecal and mucosal samples respectively. The count tables at a given taxonomic rank of the core

TABLE 1 Demographic and clinical characteristics of patients with ulcerative colitis using three different oral 5-aminosalicylic acid (5-ASA) formulations

	Mezavant	Asacol	Pentasa	P-value
Number of patients, n	18	14	10	—
Dose (g/d), median (IQR)	4.8 (0.0)	4.8 (0.2)	4.0 (0.0)	—
Gender (M-F) (n-n)	11-7	5-9	7-3	0.19
Age at diagnosis (y), mean (SD)	35.8 (9.7)	31.4 (12)	37.2 (13.7)	0.325
Duration of disease (y), median (IQR)	8.5 (5)	11.0 (6)	7.0 (15)	0.076
Disease extension, n (%)				
Rectosigmoid involvement	6 (33)	3 (22)	3 (30)	0.796
Left sided	3 (17)	1 (7)	1 (10)	
Pancolitis	9 (50)	10 (71)	6 (60)	
Previous medical therapy (≥3 mo ago), n (%)				
Prednisolone	17 (94)	14 (100)	8 (80)	—
Methotrexate	0	1 (7)	0	—
Azathioprine	1 (5.5)	2 (14)	0	—
Anti-TNF α	0	2 (14)	0	—
Current co-medication, n (%) ^a				
No co-medication	10 (56)	10 (72)	7 (70)	0.802
Laboratory values				
Hb ^b (g/dL), mean (SD)	14.4 (1.3)	13.9 (1.4)	14.9 (1.1)	0.168
CRP ^c (mg/L), median (IQR)	<5 (0)	<5 (4)	<5 (0)	0.074
ESR ^d (mm/h), median (IQR)	3.0 (4)	7.0 (12) ^e	4.5 (6)	0.210
Leukocytes ($\times 10^9/L$), mean (SD)	6.7 (1.4)	8.1 (2)	6.6 (1.1)	0.036 ^f
Faecal calprotectin (mg/kg), median (IQR)	33 (243)	93 (175)	55 (410)	0.253
Disease activity				
Total Mayo score, median (IQR)	1.5 (3.0)	1.0 (2.25)	0.5 (1.25)	0.055
Clinical Mayo score, median (IQR)	1.0 (2.0)	0.5 (2.0)	0.0 (1.0)	0.311
Endoscopic Mayo score, median (IQR)	1.0 (2.0)	0.0 (1.0)	0.0 (1.0)	0.010 ^g
Endoscopic remission ^h , n (%)	12 (67%)	13 (93%)	10 (100%)	0.021 ⁱ
Histologic Geboes score, median (IQR)	1.1 (1.1)	1.1 (0.1)	0.1 (1.3)	0.328
Histologic remission ^j , n (%)	14 (78)	11 (79)	7 (70)	0.873
Deep remission ^k , n (%)	12 (67)	11 (79)	7 (70)	0.750

^aPercentage of patients on monotherapy with oral 5-ASA and no co-medication. For patients using co-medication, the following drugs were used: amiloride/hydrochlorothiazide (n = 1), lisinopril/hydrochlorothiazide (n = 1), losartan/ hydrochlorothiazide (n = 1), calcium and cholecalciferol (n = 3), zolendronate (n = 1), alendronate (n = 2), pantoprazole (n = 1), esomeprazole (n = 1), drospirenone/ethinylestradiol (n = 1), estradiol/norethindrone acetate (n = 1), ethinylestradiol/levonorgestrel (n = 1), venlafaxine (n = 2), levothyroxine (n = 2), tiotropium bromide (n = 1), simvastatin (n = 1), cetirizine (n = 1), paracetamol (n = 1), zolmitriptan (n = 1), sumatriptan (n = 1), desoximetasone crème 2.5 mg/g (n = 1), terbinafine (n = 1).

^bBlood haemoglobin.

^cC-reactive protein.

^dPlasma erythrocyte sedimentation rate.

^eFor one Asacol patient SR-value is missing.

^fAsacol group had higher leukocyte concentration.

^gMezavant group had higher endoscopic Mayo scores.

^hDefined as a endoscopic Mayo score ≤ 1 .

ⁱMezavant patients had lower rates of endoscopic remission.

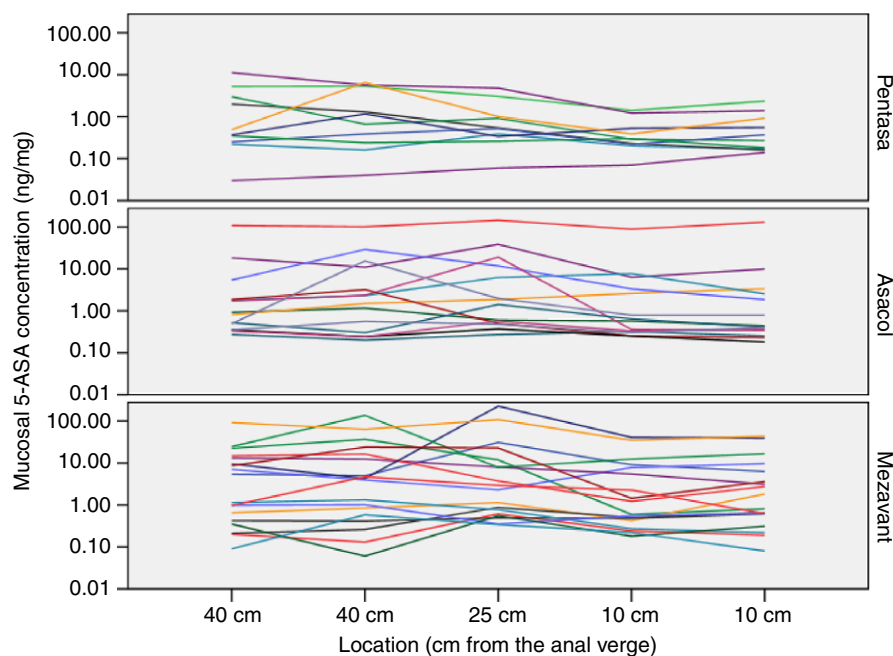
^jDefined as a Geboes score <2.1.

^kDefined as a endoscopic Mayo score ≤ 1 and a Geboes score <2.

microbiome were imported into the DESeq2 R package to estimate which OTUs that significantly correlated with log-transformed mucosal 5-ASA concentrations.³⁸ Significant OTUs were identified using a

regression model in DESeq2 using default options and *P*-values were estimated using a Wald test and adjusted for multiple testing by Benjamini Hochberg false discovery rate correction.

FIGURE 1 Mucosal 5-aminosalicylic acid (5-ASA) concentration in five colonic biopsies sampled 40, 25 and 10 cm from the anal verge in patients with ulcerative colitis using three different oral 5-ASA formulations. Each line represents one patient



3 | RESULTS

3.1 | Patient characteristics

Demographic and clinical characteristics are presented in Table 1. Overall the three 5-ASA formulation groups were similar. There were no significant differences in total Mayo score ($P = 0.055$) or proportion of patients in deep remission ($P = 0.750$). However, the Asacol group had higher leukocyte concentrations ($P = 0.036$), and the Mezavant group had higher endoscopic Mayo scores ($P = 0.01$) and fewer patients in endoscopic remission ($P = 0.021$). All Pentasa patients were classified to be in endoscopic remission, while 12 (67%) Mezavant patients and 13 (93%) Asacol patients were in endoscopic remission ($P = 0.021$). The proportion of patients in histological remission was, however, lowest in the Pentasa group, $n = 7$ (70%).

3.2 | 5-ASA drug formulation was associated with mucosal 5-ASA concentration

A graphical display of the individual 5-ASA concentrations at different locations (Figure 1) illustrates large inter-individual variations, but small intra-individual variation between segments. Boxplots of mucosal 5-ASA concentration at all locations are shown in Figure S4.

The overall test for difference in adjusted mean 5-ASA concentration (averaged over location) between the three 5-ASA formulation groups (Table 2) did not reach statistical significance ($P = 0.099$), but pairwise comparisons revealed a significant higher mean concentration in patients using Mezavant compared with Pentasa (geometric mean 2.39 ng/mg vs 0.57 ng/mg, $P = 0.033$) and a nonsignificant higher mean value compared to the Asacol group (2.39 ng/mg vs 1.60 ng/mg, $P = 0.50$). Recalculated into % difference, the Pentasa patients had 76% lower 5-ASA concentrations compared with

Mezavant patients, and Asacol patients had in average 33% lower concentrations. The mean 5-ASA concentration level differed significantly by location ($P \leq 0.001$), with the lowest concentrations in the rectum regardless of formulation. Despite a more clear decreasing trend according to location in the Pentasa group, the test for interaction between location and formulation did not reach statistical significance ($P = 0.68$). Consequently, the difference between the three 5-ASA formulations was consistently observed at each location, although power of test within subgroups was low. Adjustment for deep remission did not change the results notably, but provided more precise estimates (slightly lower P -values and smaller CI, results not shown).

The mucosal concentration of Ac-5-ASA decreased significantly from oral to anal direction, and did not differ between the formulations overall ($P = 0.47$) or in pairwise comparisons (Mezavant vs Pentasa $P = 0.23$ and Mezavant vs Asacol $P = 0.48$). There were no significant differences in serum 5-ASA ($P = 0.20$) or serum Ac-5-ASA ($P = 0.20$) between the different 5-ASA formulations (Table 3).

3.3 | Mucosal 5-ASA concentration was positively associated to abundance of numerous beneficial bacteria

Mucosal 5-ASA concentration was significantly associated to the mucosal bacterial abundance on all taxonomic levels (Table 4), whereas the association to bacterial abundance in faecal samples was weaker (Table 5). Mucosal 5-ASA concentration and alpha-diversity was positively associated in mucosal biopsies ($P = 4.7 \times 10^{-4}$), whereas no association was found in faecal samples (Figure 2). Regression analysis of bacterial abundances on phylum level and mucosal 5-ASA concentration (Figure 3) revealed that mucosal 5-ASA mucosal concentrations were associated with lower abundances

TABLE 2 Mucosal concentrations (ng/mg) of 5-aminosalicylic acid (5-ASA) and acetylated 5-ASA (Ac-5-ASA) in the left hemicolon and rectum in patients with ulcerative colitis using three different oral 5-ASA formulations

	Geometric mean (95% confidence interval) ^a			P-value	
	Mezavant (n = 18)	Asacol (n = 14)	Pentasa (n = 10)	By location ^b	By formulation ^c
5-ASA concentrations					
Adjusted mean ^d	2.39 (1.09-5.28)	1.60 (0.65-3.91)	0.57 (0.20-1.64)	0.099	
Difference (%)	1.0 (reference)	-33.2 (-79.8, 119.7)	-76.3 (-93.7, -11.2)		
P-value ^e		0.50	0.033		
By location (cm from the anal verge)				<0.001	
40	2.71 (1.20-6.10)	1.71 (0.68-4.31)	0.76 (0.26-2.26)	0.24	
25	3.22 (1.36-7.59)	2.31 (0.88-6.10)	0.58 (0.18-1.81)	0.064	
10	1.61 (0.71-3.63)	1.09 (0.43-2.74)	0.37 (0.13-1.10)	0.081	
P-value, location ^f	0.013	0.011	0.006		
P-value, interaction ^g	0.68				
Ac-5-ASA concentrations					
Adjusted mean ^d	1.56 (0.98-2.49)	1.03 (0.61-1.74)	1.19 (0.64-2.22)	0.47	
Difference (%)	1.0 (reference)	-34.3 (-67.4, 32.4)	-23.9(-64.9, 65.2)		
P-value ^e		0.23	0.48		
By location (cm from the anal verge):				<0.001	
40	2.03 (1.24-3.30)	1.68 (0.97-2.93)	2.02 (1.05-3.89)	0.85	
25	1.50 (0.88-2.57)	0.88 (0.48-1.61)	1.05 (0.52-2.15)	0.47	
10	1.29 (0.79-2.10)	0.71 (0.41-1.24)	0.78 (0.40-1.50)	0.27	
P-value, location ^f	0.009	<0.001	<0.001		
P-value, interaction ^g	0.23				

^aResults based on analyses of log-transformed data in multilevel linear mixed model, with and without interaction between 5-ASA formulation type and location, and with additional adjustments for pinch biopsy site and replicates (replicate samples at 40 and 10 cm).

^bF-tests for difference in mean values between locations; 10, 25 and 40 cm from the anal verge (no-interaction model).

^cF-tests for difference in mean values between 5-ASA formulation groups, overall (no-interaction model) and at each specific location (interaction model).

^dOverall mean, averaged over location and order of sampling (estimated marginal means from no-interaction model).

^ePairwise tests (each group compared with Mezavant).

^fF-tests for difference in mean values between locations within each 5-ASA formulation (interaction model).

^gF-test for interaction between location and 5-ASA formulation type.

TABLE 3 Serum concentrations (ng/mL) of 5-aminosalicylic acid (5-ASA) and acetylated 5-ASA (Ac-5-ASA) in patients with ulcerative colitis using three different oral 5-ASA formulations

	Mezavant	Asacol	Pentasa	P-value ^a
Serum concentrations (ng/mL), median (interquartile range)				
5-ASA	3420 (5085)	1658 (3347)	3028 (6317)	0.202
Ac-5-ASA	2319 (2769)	1770 (2439)	4634 (5555)	0.200

^aCompared using Kruskal-Wallis test.

of the Proteobacteria phylum ($P = 1.2 \times 10^{-15}$) and higher abundances of Firmicutes ($P = 2.6 \times 10^{-6}$) and Bacteroidetes ($P = 3.1 \times 10^{-4}$) in mucosal biopsies. There were no associations between faecal bacterial abundance on phylum level and 5-ASA mucosal concentration.

In the mucosal biopsies, 10 bacterial families were positively and six families were negatively associated to mucosal 5-ASA

concentration (Figures S5 and S6), the most significant association was seen for Lachnospiraceae and Ruminococcaceae families (Figure 4A,B). Totally 19 mucosal bacterial genera were associated with 5-ASA concentration. The bacterial genus in the mucosa that was most significantly associated to 5-ASA concentration was *Faecalibacterium* (4.8×10^{-16}) (Figure 4C), followed by the genera *Blautia* (Figure 4D), *Bacteroides*, *Coprococcus* and *Dorea*. Of the genera associated with 5-ASA concentration, 18 of 19 were positively associated (Figure S7). *Cloacibacterium* was the only genus negatively associated ($P = 0.048$) with mucosal 5-ASA concentration in the mucosal biopsies (Figure 4F). On species level, *Faecalibacterium prausnitzii* was positively correlated with mucosal 5-ASA concentration ($P = 1.3 \times 10^{-6}$) (Figure 4E). In faeces, two bacterial genera were associated with 5-ASA mucosal concentration; *Prevotella* ($P = 7.2 \times 10^{-5}$) and *Sutterella* ($P = 0.03$), both genera were negatively associated with 5-ASA concentrations (Table 5).

TABLE 4 Mucosal bacteria abundances significantly associated with 5-aminosalicylic acid (5-ASA) concentration on different taxonomic levels (regression analysis)

Phylum	Class	Order	Family	Genus	P	B
Proteobacteria	—	—	—	—	1.2×10^{-15}	-1.29
Firmicutes	—	—	—	—	2.6×10^{-6}	0.48
Bacteroidetes	—	—	—	—	3.1×10^{-4}	0.99
Firmicutes	Clostridia	—	—	—	6.1×10^{-21}	2.33
Bacteroidetes	Bacteroidia	—	—	—	6.9×10^{-9}	2.10
Proteobacteria	Alphaproteobacteria	—	—	—	1.2×10^{-6}	-0.87
Firmicutes	Bacilli	—	—	—	1.2×10^{-5}	-0.70
Proteobacteria	Betaproteobacteria	—	—	—	5.5×10^{-5}	-0.65
Bacteroidetes	Flavobacteriia	—	—	—	4.8×10^{-3}	-2.34
Firmicutes	Clostridia	Clostridiales	—	—	2.4×10^{-20}	2.62
Bacteroidetes	Bacteroidia	Bacteroidales	—	—	8.2×10^{-10}	2.10
Actinobacteria	Actinobacteria	Bifidobacteriales	—	—	1.3×10^{-4}	1.94
Firmicutes	Bacilli	Lactobacillales	—	—	1.4×10^{-4}	3.37
Proteobacteria	Alphaproteobacteria	Rhizobiales	—	—	9.8×10^{-4}	-0.57
Firmicutes	Bacilli	Bacillales	—	—	0.002	-0.49
Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	—	7.8×10^{-13}	1.73
Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	—	1.6×10^{-12}	2.01
Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	—	9.8×10^{-12}	-1.27
Firmicutes	Bacilli	Bacilliales	Bacillaceae	—	6.2×10^{-12}	-1.32
Proteobacteria	Alphaproteobacteria	Rhizobiales	Bruceellaceae	—	2.8×10^{-8}	-1.51
Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae	—	3.9×10^{-5}	1.46
Bacteroidetes	Flavobacteriia	Flavobacteriales	Weeksellaceae	—	5.7×10^{-4}	-2.95
Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae	—	8.2×10^{-4}	2.04
Bacteroidetes	Bacteroidia	Bacteroidales	Rikenellaceae	—	0.011	2.38
Proteobacteria	Alphaproteobacteria	Rhizobiales	Methylobacteriaceae	—	0.015	-1.11
Proteobacteria	Betaproteobacteria	Burkholderiales	Alcaligenaceae	—	0.015	2.10
Firmicutes	Clostridia	Clostridiales	Clostridiaceae	—	0.015	1.76
Actinobacteria	Actinobacteria	Actinomycetales	Microbacteriaceae	—	0.029	-0.74
Bacteroidetes	Bacteroidia	Bacteroidales	Prevotellaceae	—	0.031	3.60
Actinobacteria	Actinobacteria	Bifidobacteriales	Bifidobacteriaceae	—	0.031	0.99
Firmicutes	Bacilli	Lactobacillales	Streptococcaceae	—	0.035	1.87
Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	Faecalibacterium	4.8×10^{-16}	3.16
Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Blautia	2.1×10^{-14}	2.60
Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae	Bacteroides	1.8×10^{-10}	2.72
Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Coproccoccus	3.6×10^{-10}	2.41
Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Dorea	1.8×10^{-8}	3.05
Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Ruminococcus	6.1×10^{-8}	3.97
Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	Oscillospira	6.2×10^{-8}	3.94
Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Roseburia	9.6×10^{-8}	3.39
Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae	Parabacteroides	1.0×10^{-7}	3.40
Actinobacteria	Actinobacteria	Bifidobacteriales	Bifidobacteriaceae	Bifidobacterium	1.9×10^{-5}	2.10
Firmicutes	Bacilli	Lactobacillales	Streptococcaceae	Streptococcus	5.5×10^{-5}	3.55
Proteobacteria	Betaproteobacteria	Burkholderiales	Alcaligenaceae	Sutterella	1.2×10^{-4}	3.36
Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Lachnospira	1.2×10^{-4}	2.02
Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	Ruminococcus	2.1×10^{-4}	2.76

(Continues)

TABLE 4 (Continued)

Phylum	Class	Order	Family	Genus	P	B
Actinobacteria	Coriobacteriia	Coriobacteriales	Coriobacteriaceae	Collinsella	1.2×10^{-3}	2.17
Bacteroidetes	Bacteroidia	Bacteroidales	Odoribacteraceae	Odoribacter	9.3×10^{-3}	2.48
Bacteroidetes	Bacteroidia	Bacteroidales	Prevotellaceae	Prevotella	0.021	4.51
Firmicutes	Clostridia	Clostridiales	Veillonellaceae	Dialister	0.028	1.40
Bacteroidetes	Bacteroidia	Bacteroidales	Weeksellaceae	Cloacibacterium	0.048	-1.62

Bacteria within each taxonomic level are listed by increasing *P*-values. *P* = adjusted *P*-value, *B* = regression coefficient. Red text = increased abundance (positive association), blue = decreased abundance (negative association).

TABLE 5 Faecal bacteria abundances significantly associated with 5-aminosalicylic acid (5-ASA) concentration on different taxonomic levels (regression analysis)

Phylum	Class	Order	Family	Genus	P	B
Proteobacteria	Betaproteobacteria	—	—	—	0.004	-2.02
Bacteroidetes	Betaproteobacteria	Burkholderiales	—	—	0.007	-2.02
Bacteroidetes	Bacteroidia	Bacteroidales	Prevotellaceae	—	7.7×10^{-5}	-5.17
Firmicutes	Betaproteobacteria	Burkholderiales	Alcaligenaceae	—	0.02	-1.92
Bacteroidetes	Bacteroidia	Bacteroidales	Prevotellaceae	Prevotella	7.2×10^{-5}	-5.27
Firmicutes	Betaproteobacteria	Burkholderiales	Alcaligenaceae	Sutterella	0.025	-1.91

Bacteria within each taxonomic level are listed by increasing *P*-values. *P* = adjusted *P*-value, *B* = regression coefficient, Blue text = decreased abundance (negative association).

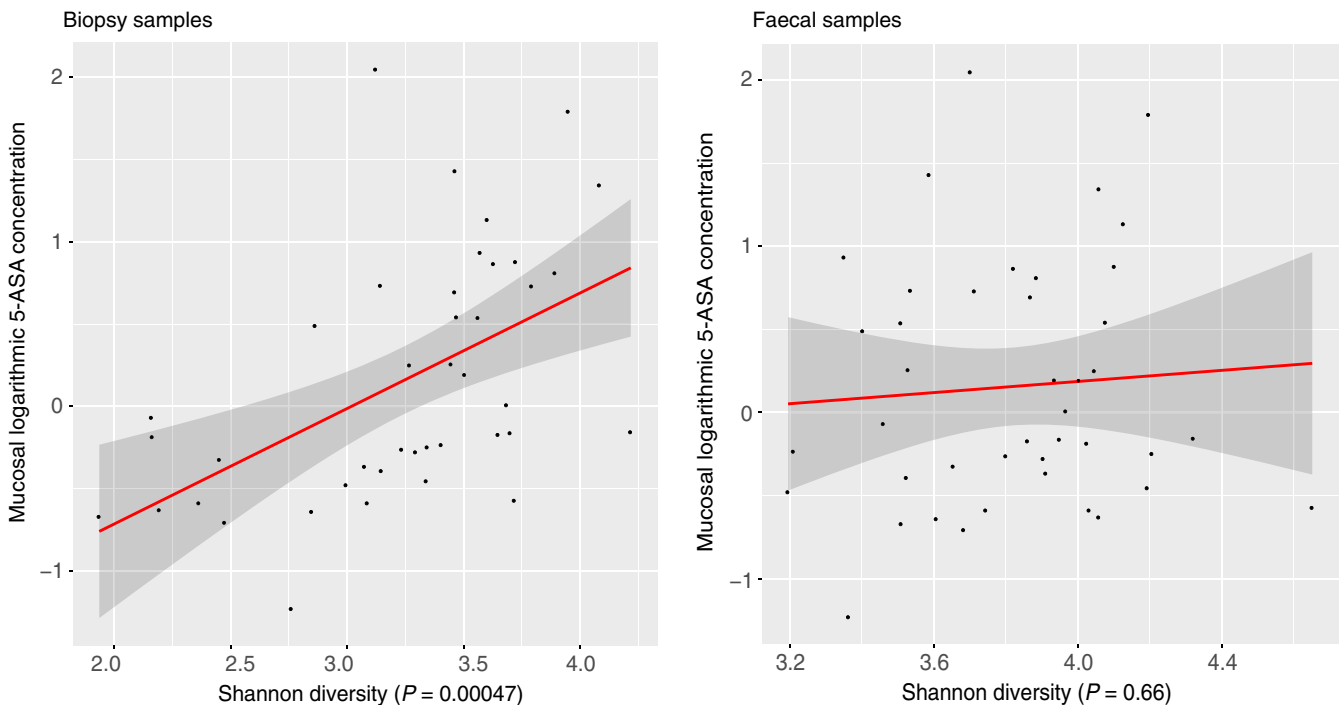


FIGURE 2 Bacterial diversity analysis in biopsies and faecal samples in patients with ulcerative colitis using 5-aminosalicylic acid (5-ASA). Red line = regression line, dark grey area = 95% confidence interval

3.4 | Mucosal 5-ASA concentration was not associated with mucosal inflammation

Mucosal 5-ASA concentrations and disease activity were not significantly associated in our patients. Mucosal 5-ASA concentration was

neither associated with deep remission ($P = 0.106$), total Mayo score ($P = 0.114$) nor endoscopic Mayo score ($P = 0.055$). The inflammation variables tended to be higher in patients with high mucosal 5-ASA concentration.

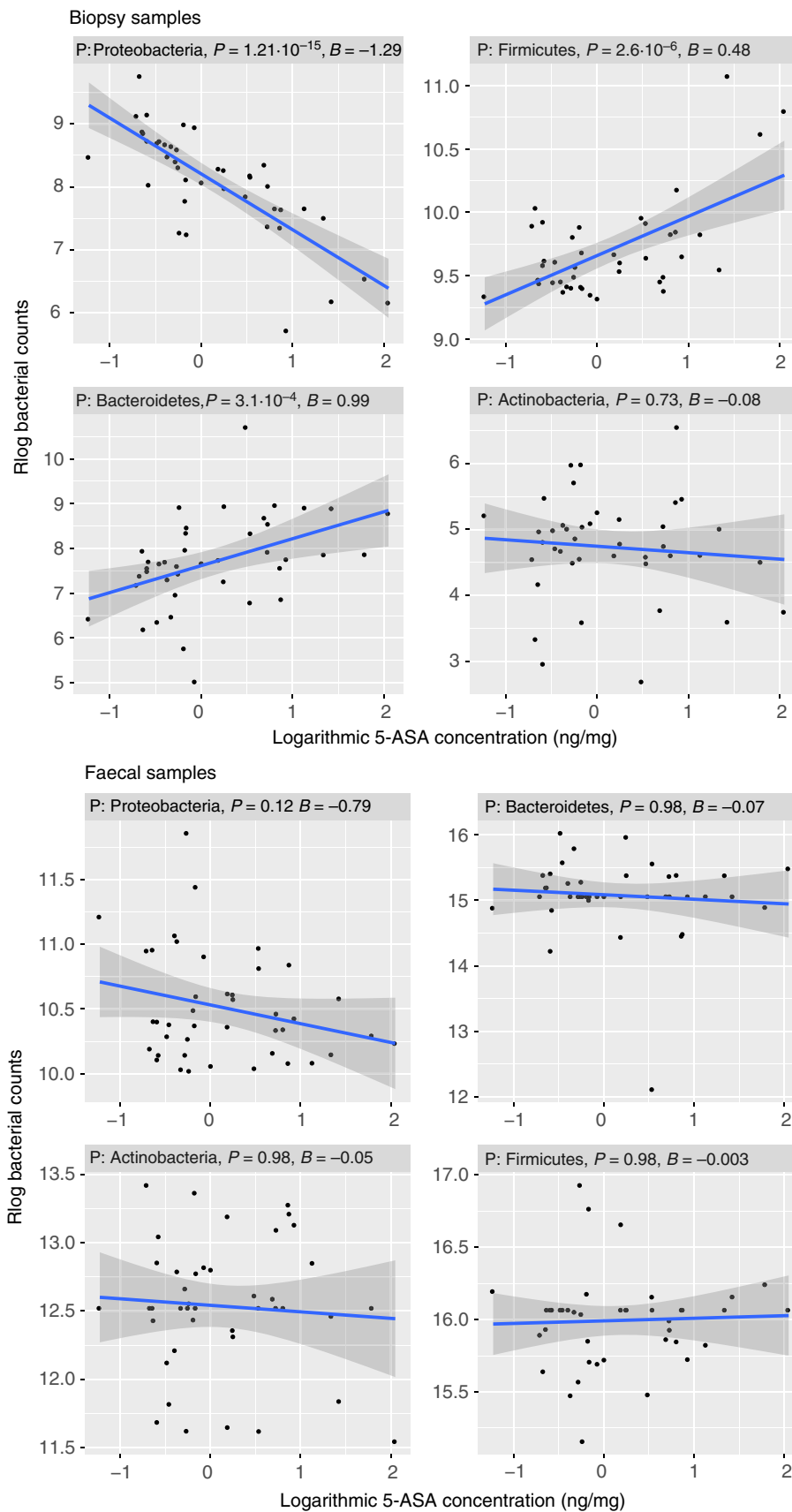


FIGURE 3 Bacterial phyla correlated to 5-aminosalicylic acid (5-ASA) mucosal concentration in biopsies and faecal samples in patients with ulcerative colitis (UC). P: phylum, P = adjusted P -value, B = regression coefficient, Rlog = regularised logarithm transformation. Blue line = regression line. Dark grey area = 95% confidence interval

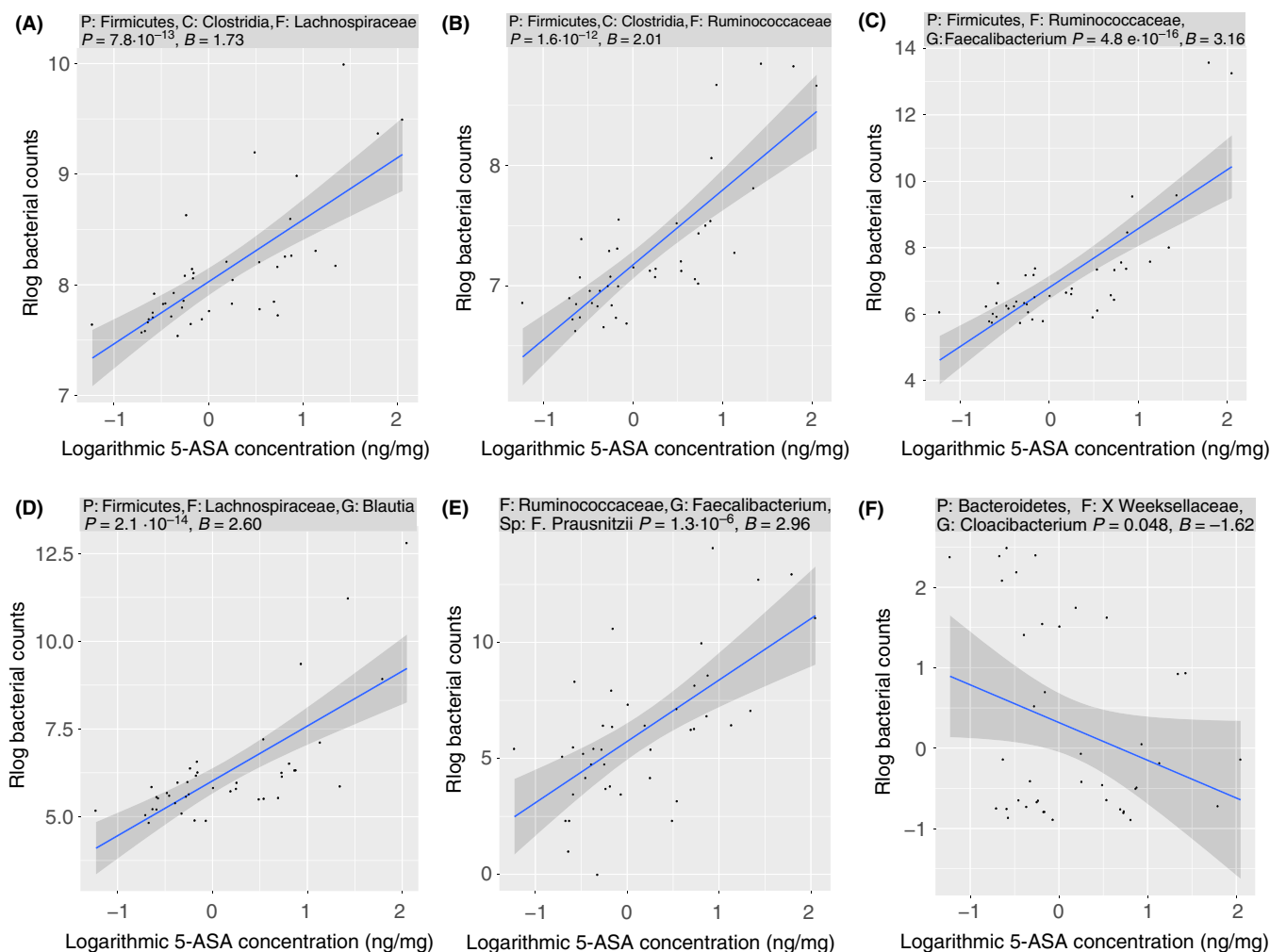


FIGURE 4 Selected bacterial families, genera and species significantly correlated to 5-aminosalicylic acid (5-ASA) mucosal concentration in mucosal biopsies in patients with ulcerative colitis; (A) Lachnospiraceae, (B) Ruminococcaceae, (C) Faecalibacterium, (D) Blautia, (E) Faecalibacterium prausnitzii, (F) Cloacibacterium (for complete list see Figures S5–S7). P, phylum; C, class; F, family; G, genus; Sp, species. P = adjusted P -value; B = regression coefficient, Rlog = regularised logarithm transformation. Blue line = regression line. Dark grey area = 95% confidence interval

3.5 | NAT1 genotype influences serum, but not mucosal 5-ASA concentration

All patients were successfully genotyped for NAT1 and NAT2. Eight patients (19.1%) had a slow acetylator NAT1 phenotype (Table 6). Thirty-one patients (73.8%) had a slow acetylator NAT2 phenotype (Table S3). Patients with NAT1 slow acetylators status had significantly higher 5-ASA serum concentrations ($P = 0.044$) than patients with NAT1 rapid acetylator status. There was no significant association between serum 5-ASA concentration and NAT2 genotype ($P = 0.708$). Mucosal 5-ASA concentration was not significantly associated with neither NAT1 ($P = 0.276$) nor NAT2 ($P = 0.488$) genotype.

4 | DISCUSSION

This is the first study to measure mucosal 5-ASA concentration in UC patients using different oral 5-ASA formulations, and correlating

it to NAT genotype and bacterial microbiota. Concurring with previous findings, our patients had declining concentrations of 5-ASA towards the rectum.¹⁶ A novel finding was that patients using Mezavant had higher overall mucosal 5-ASA concentrations, compared to patients using Pentasa, while there was no significant difference between patients using Mezavant and Asacol. All patients used high doses of 5-ASA, however the manufacturers' maximal recommended dose of Pentasa (4.0 g) is 17% lower than of Mezavant (4.8 g) and Asacol (4.8 g), but this could only explain a minor proportion of the observed differences. The large inter-individual variation in concentration was consistent with previous reports,^{4–7} whereas the intra-individual variation between bowel segments was small. Factors such as colonic transit time, intraluminal pH and disease pattern may affect dissolution and uptake of 5-ASA in the colon³⁹ and could explain some of the inter-individual variation in 5-ASA concentration reported in the present and previous studies.^{4,15,16} The mucosal Ac-5-ASA concentration reflects the amount of 5-ASA metabolised by NAT1 in the colonic mucosa. There were no significant differences

TABLE 6 Frequencies of N-acetyl-transferase 1 (NAT1) genotypes and phenotype/acetylator status in patients with ulcerative colitis using oral 5-aminosalicylic acid

NAT1 Genotype	Number of patients (n)	Phenotype/Acetylator status	Observed frequency (%)
NAT1*3/*4	1	Rapid	2.4
NAT1*4/*4	24	Rapid	57.1
NAT1*4/*10	7	Rapid	16.7
NAT1*4/*14B	2	Slow	4.8
NAT1*4/*15	1	Slow	2.4
NAT1*4/*16	1	Rapid	2.4
NAT1*4/*17	4	Slow	9.5
NAT1*10/*10	1	Rapid	2.4
NAT1*10/*14A	1	Slow	2.4

in Ac-5-ASA mucosal concentrations between the three 5-ASA formulations. Similarly, the serum concentrations of 5-ASA or Ac-5-ASA did not differ between the formulations.

The intestinal bacterial composition and metabolic products may affect disease activity in UC. 5-ASA has been found to inhibit in vitro growth of *Mycobacterium avium* paratuberculosis as well as anaerobic bacteria.^{31,40} 5-ASA also influence bacterial gene expression causing reduced bacterial invasiveness.³⁰ Furthermore, in patients with irritable bowel syndrome, 5-ASA reduces overall faecal bacterial abundance, increases Firmicutes and decreases Bacteroidetes abundances.²⁹ In the present study, mucosal 5-ASA concentration was remarkably associated with alterations in the mucosal bacterial composition, but to a lesser degree with alterations in faecal microbiota. High mucosal 5-ASA concentration was associated with high bacterial diversity, decreased mucosal abundances of Proteobacteria phylum and increased mucosal abundances of Firmicutes and Bacteroidetes phyla. Reduced bacterial diversity is a hallmark of dysbiosis in UC, whereas increased bacterial diversity is perceived beneficial.^{20,21,25,41} Proteobacteria are increased in patients with UC,^{25,42,43} and linked to increased disease activity and relapse frequency.^{24,42} Overall, the bacterial genera positively associated with high 5-ASA concentrations have previously been associated with beneficial effects in IBD: (a) the abundances of the butyrate producing *Faecalibacterium* and *Roseburia* have been inversely correlated to disease activity in UC.⁴⁴ (b) *Blautia*, *Bacteroides*, *Parabacteroides* and *Sutterella* have all been reported to be inversely correlated with inflammation in patients with ileal-pouch anal anastomosis.⁴⁵ (c) Ruminococcaceae and *Ruminococcus* spp in donor faeces transplanted to UC patients is associated with induction of remission⁴⁶ and (d) the Lachnospiraceae and Ruminococcaceae family, to which most genera in Table 4 belong, may protect against UC as they are increased in healthy twins discordant for UC.²² One genus, *Cloacibacterium*, was found to be negatively associated to mucosal 5-ASA concentration, recently this genus has been reported to be more abundant in inflamed vs. non-inflamed tissue of UC patients.²³

Although 16S rRNA sequencing may not be the preferred method for analysing bacteria on species level, the mucosal 5-ASA

concentration was positively associated with the mucosal abundance of *F. prausnitzii*. *Faecalibacterium prausnitzii* is found to be depleted in UC patients and inversely correlated with disease activity, whereas increased abundance is associated with long-term remission.^{44,47}

The mucosal 5-ASA concentration was not associated with alterations in the faecal bacteriome to the same extent as the mucosal bacteriome. The faecal bacterial diversity, and bacterial abundance on phylum level, were not associated with mucosal 5-ASA concentrations. However, abundances of the Betaproteobacteria class and Burkholderiales order in faeces were negatively associated to mucosal 5-ASA concentration, the former association was also found in the mucosa and is presumed favourable in UC.^{24,42}

5-ASA concentration have previously been found to be inversely correlated to disease activity index scores,^{5,7} endoscopic and histologic remission^{4,6} in UC patients with mild to moderate disease activity. In the present study, patients had low disease activity, and in contrast to previous findings, the 5-ASA concentration was not significantly associated with the combined parameters of disease activity. Such associations may not be evident within a relatively homogenous patient group with low disease activity. In fact, there was a trend towards higher mucosal 5-ASA concentrations in patients with higher endoscopic scores and it is unlikely that the microbiome features associated with mucosal 5-ASA concentration were confounded by the degree of inflammation. NAT1 and NAT2 genotypes have previously not been found to influence treatment efficacy,⁴⁸ but studies correlating NAT genotypes to 5-ASA mucosal concentrations have previously not been published. We found no correlation between NAT genotypes and mucosal 5-ASA concentration, thus NAT1 genotype cannot explain the large inter-individual variations in mucosal 5-ASA concentrations in our study. Interestingly we found that patients with the NAT1 slow acetylator genotype had significantly higher serum 5-ASA concentrations compared to patients with NAT1 rapid acetylator genotype, which could theoretically influence the risk of concentration dependent systemic adverse effects.

5 | CONCLUSIONS

Patients using Mezavant had significantly higher mucosal 5-ASA concentrations than patients using Pentasa. Our results suggest that 5-ASA increases bacterial diversity, favours numerous beneficial bacteria and inhibits disadvantageous bacteria in UC patients. In conclusion, our novel findings indicate that 5-ASA may have positive effects on the mucosal microbiome and could amend dysbiosis in UC patients.

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AUTHORSHIP

Guarantor of the article: Reidar Fossmark.

Author contributions: TCM and RF designed the study. MO, TCM and RF have collected the data. OS, AF, WRB, GA, ESR, and BG have contributed to analyses of biological material and data. MO, OS, AF, AvBG, GA, AKS, TCM and RF have interpreted the results and drafted the manuscript. All authors have approved the final version of the manuscript including the author list.

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