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Analysis of Volatile Organic Compounds of Bacterial Origin in Chronic Gastrointestinal Diseases

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Background: The aim of this study was to determine whether volatile organic compounds (VOCs) present in the headspace of feces could be used to diagnose or distinguish between chronic diseases of the gastrointestinal tract and apparently healthy volunteers.

Methods: A total of 87 people were recruited, divided between 4 categories: healthy volunteers (n = 19), Crohn's disease (n = 22), ulcerative colitis (n = 20), and irritable bowel syndrome (n = 26). They each supplied fecal samples before, and except for the healthy volunteers, after treatment. Fecal samples were incubated in a sample bag with added purified air at 40°C and headspace samples were taken and concentrated on thermal sorption tubes. Gas chromatography–mass spectrometry then desorbed and analyzed these. The concentrations of a selection of high-abundance compounds were determined and assessed for differences in concentration between the groups.

Results: Crohn's disease samples showed significant elevations in the concentrations of ester and alcohol derivates of short-chain fatty acids and indole compared with the other groups; indole and phenol were elevated in ulcerative colitis and irritable bowel syndrome but not at a statistically significant level. After treatment, the levels of many of the VOCs were significantly reduced and were more similar to those concentrations in healthy controls.

Conclusions: The abundance of a number of VOCs in feces differs markedly between Crohn's disease and other gastrointestinal conditions. Following treatment, the VOC profile is altered to more closely resemble that of healthy volunteers.

(Inflamm Bowel Dis 2013;0:1–10)

Key Words: Crohn's disease, colitis, ulcerative, irritable bowel syndrome, fatty acids, volatile, volatile organic compounds

Chronic diseases of the intestinal tract present considerable practical difficulties, both in terms of diagnosis and of health economics. Irritable bowel syndrome (IBS) is the most common gastrointestinal (GI) disorder encountered in the Western world, affecting perhaps 15% of the population yet remains a diagnosis of exclusion. A series of expensive and sometimes unpleasant investigations, including analyses of blood, urine and feces, radiology, and endoscopy, must be performed to exclude other more dangerous diseases, before the diagnosis can be accepted. Inflammatory bowel disease (IBD) is less common but presents with symptoms of abdominal pain and diarrhea that might be very similar to those of IBS. Diverticular disease and carcinoma of the colon must also be excluded.

In recent years, growing attention has been focused on the role of the intestinal microbiota in the pathogenesis of chronic GI diseases.[1] The intestinal microbiota, however, is very complex, ~~with~~ 10^{11}–10^{12} bacteria per gram of contents present in the colon.[2] A report from the MetaHIT (Metagenomics of the Human Intestinal Tract) project estimated that a cohort of 124 European volunteers harbored a total some of 1150 distinct bacterial species, with each individual having at least 160.[3] As yet, no specific pathogen has been shown to be associated with any of these disorders. The microbiota is known, however, to be abnormal in both IBS and IBD, with a reduction in the number of Actinobacteria and Firmicutes and overgrowth by Proteobacteria.[4–6]

The microbiota has been shown to be the subject of immune attack in IBD.[7] Over 80% of fecal microorganisms are coated with immunoglobulin, but these numbers fall rapidly in patients receiving successful treatment.[8] A role for the microbiota has also been shown in IBS with abnormal bacterial fermentation, leading to increased excretion of hydrogen. Correction of such fermentation whether by antibiotics or diet leads to amelioration of symptoms,[9–11] and abnormal fermentation is now believed to underlie reports of food intolerances in some cases of IBS.[12,13]

Progress in chemometrics means that it is now possible to analyze complex mixtures of chemical compounds in biological

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fluids.[14] There is strong anecdotal evidence that feces from patients with GI disease have an abnormal smell, moreover, that different malodors are indicative of particular conditions. It has long been known that bacteria emit volatile organic compounds (VOCs) as by-products of metabolism. Kuzma et al[15] demonstrated the production of isoprene from gram-positive and gram-negative species, whereas later studies showed, for example, the production of hydrogen cyanide from *Pseudomonas aeruginosa*,[16,17] methanethiol and ammonia from methionine in *Clostridium sporogenes*, and indole and phenol as products of aromatic amino acid breakdown.[18] A number of reports have suggested that bacteria may be distinguished by means of their individual VOC profiles and that it might prove possible to develop this approach for diagnostic purposes.[19–21] Several studies that have used a variety of analytical approaches to differentiate between infected and noninfected samples in a range of biological matrices such as urine,[22] sputum,[23] and feces supported these findings.[24]

This suggests that different patterns of VOC production in fecal samples may indicate variations in the gut microbiota, which in turn are indicative of specific disease processes. If so, determination of fecal VOC profiles could offer a route to the rapid and noninvasive diagnosis of a range of GI diseases. Studies of patients with ulcerative colitis (UC), *Clostridium difficile*, and *Campylobacter jejuni* infections, which have yielded encouraging results, supported this idea.[25,26] These studies used solid-phase extraction of fecal headspace VOCs followed by analysis using gas chromatography–mass spectrometry to establish presence/absence scores for over 100 volatile species and demonstrated that different patterns of VOCs were associated with each condition investigated.

In this study, we have extended this approach by using pumped headspace sampling onto thermal desorption (TD) tubes, which has allowed us to make quantitative estimations of the concentration of VOCs evolved from human fecal samples. We have determined VOC concentration in patients with clinical diagnoses of UC, Crohn's disease (CD), or IBS before and after treatment and in a group of healthy controls. Our hypothesis is that treatment should, and ameliorating symptoms, result in a normalization of the fecal headspace VOC profile.

MATERIALS AND METHODS

Patients and Sample Collection

Patients and healthy volunteers were recruited at the Department of Gastroenterology, Addenbrooke's Hospital, Cambridge, United Kingdom. The diagnosis of CD, UC, or IBS was made by standard diagnostic criteria. All patients had symptoms of active relapse of disease. None had received previous dietary treatment, but some were taking medication including 5-aminosalicylic acid compounds and or azathioprine, which was insufficient to control their symptoms. Antibiotic usage in the previous 6 weeks was an exclusion criterion. A numerical code identified subjects and their diagnosis, and the diagnosis was not revealed to the analysts until all specimens had been tested. All signed informed consent to the procedures. Nonfasting morning samples of feces were obtained from each subject (pretreatment samples). Numbers of males and females in each of the disease and control groups are given in Table 1.

TABLE 1. Numbers of Volunteers in Disease and Control Groups

Group	Males	Females
Control	11	8
IBS	4	22
UC	12	8
CD	8	14

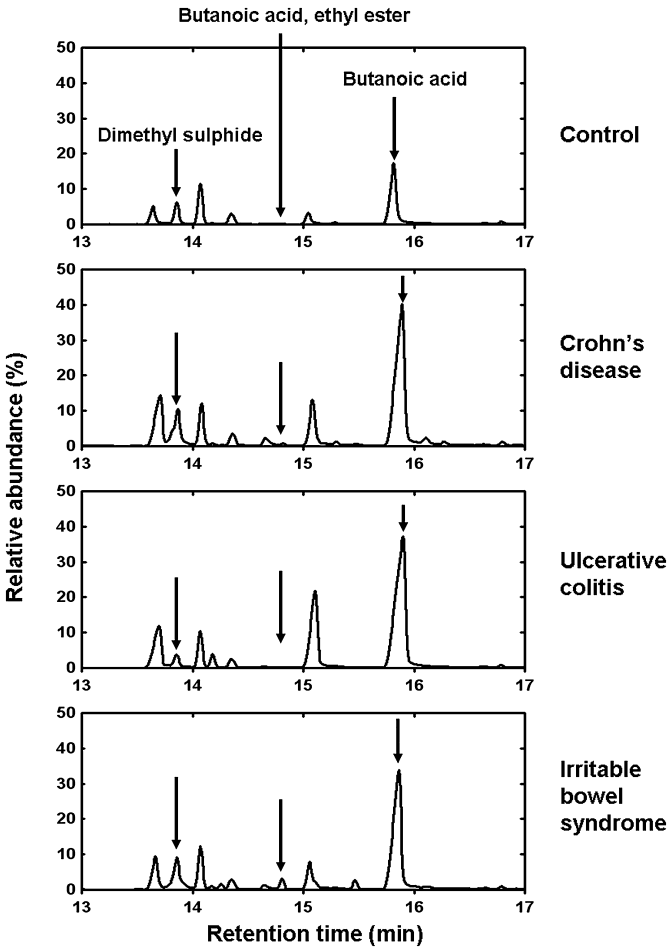

FIGURE 1. Part of a total ion chromatogram alignment for control and pretreatment disease groups, illustrating the visual inspection method for generating a list of candidate compounds for statistical analysis. Some compounds exhibiting differences in abundance between groups are indicated. The total duration of the analysis was 30 minutes, but for clarity, only the central portion (13–17 minutes) of the overall chromatogram is shown. Horizontal scale = time; vertical scale = relative abundance.

AU1

Treatment varied according to diagnostic category. Those with Crohn's disease were offered 2 weeks of treatment with an elemental feed (E028 Extra; Nutricia, Liverpool, United Kingdom).[27] Patients with UC were treated with oral corticosteroids and 5-aminosalicylic acid derivatives and those with IBS were given exclusion diets.[28] In each case, patients returned to the hospital after 2 weeks of treatment and a fecal sample was collected (posttreatment samples). Healthy control subjects attended the department on a single occasion for collection of a similar specimen. Volunteers unable to provide a fecal specimen at initial interview were given a sample pot to take away and instructed to provide a stool specimen within 48 hours and not to change their treatment in any way until this had been provided. Samples were delivered to the hospital on the same day as they were passed with a maximum delay before freezing of 4 hours. Samples were stored at −40°C until transfer to the laboratory.[29] Ethical permission for this study was granted by the Leeds West Local Ethical Committee (Ref. 07/Q1205/39).

Sample Preparation

Samples were transferred to the laboratory packed in dry ice inside insulated containers and on arrival were stored at −80°C until analyzed. Gas sampling bags for headspace analysis were prepared from 65-mm-wide Nalophan NA tube cut to 500 mm lengths. A 70 mm length of 6-mm-diameter polypropylene tubing bearing a 1/4″ Swagelok fitting was secured to one end using nylon ties. Each sample was defrosted at room temperature before analysis, a 5-mL aliquot being added to the open end of the bag using the scoop provided with the sample container. The bag was then sealed using nylon ties, and the bag was filled with hydrocarbon-free air. Bags containing samples were incubated at 40°C for 10 minutes before 500 mL of the headspace gas was pumped (100 mL/min for 5 minutes using a portable air pump) over TD tubes prepacked with 50% Carbotrap

TABLE 2. Candidate Compounds for Statistical Analysis Selected as Described in Methods

Selection Method	Name	CAS No.	RT, min
Most abundant	Acetone	67-64-1	7.23
	Propanoic acid	79-09-4	13.75
	Butanoic acid	107-92-6	15.86
Published data	1-Propanol	71-23-8	9.06
	Propanoic acid, ethyl ester	105-37-3	12.50
	Butanoic acid, methyl ester	623-42-7	12.87
	Butanoic acid, ethyl ester	105-54-4	14.85
	P-cresol	106-44-5	22.82
	Indole	120-72-9	29.49
	Dimethyl disulphide	624-92-0	13.90
Visual inspection	1-Butanol	71-36-3	11.98
	Butanoic acid, 3-methyl	503-74-2	17.08
	Phenol	108-95-2	20.90

RTs indicated are typical.
CAS number, Chemical Abstracts Service Reference Number; RT, retention time.

TABLE 3. Concentrations of VOCs in Fecal Headspace From Healthy Controls and Volunteers Diagnosed With CD, UC, and IBS Before Prescribed Treatment

	VOC Concentration, ng/L				
	Median (Lower Quartile, Upper Quartile)				
Compound	Control	CD	UC	IBS	*P*
Acetone	142 (21, 336)	83 (38, 253)	121 (80, 241)	68 (36, 151)	0.594
Propanoic acid	6 (0, 55)	183 (0, 354)	32 (0, 353)	80 (0, 303)	0.474
Butanoic acid	33 (3, 202)	1074 (22, 1521)	169 (4, 1339)	628 (83, 1362)	0.043[a]
1-Propanol	13 (0, 33)	281 (38, 897)	64 (7, 659)	33 (4, 62)	0.003[a]
Propanoic acid, ethyl ester	0 (0, 0)	30 (1, 181)	0 (0, 60)	0 (0, 4)	0.000[a]
Butanoic acid, methyl ester	0 (0, 3)	30 (6, 114)	7 (0, 79)	11 (0, 33)	0.015[a]
Butanoic acid, ethyl ester	0 (0, 1)	46 (4, 376)	22 (0, 324)	1 (0, 42)	0.006[a]
P-cresol	122 (50, 433)	499 (115, 1217)	221 (117, 960)	213 (18, 587)	0.155
Indole	9 (3, 34)	127 (56, 587)	27 (13, 163)	41 (8, 117)	0.001[a]
Dimethyl disulphide	30 (4, 71)	114 (58, 870)	71 (27, 143)	57 (11, 412)	0.075
1-Butanol	9 (0, 39)	142 (76, 406)	25 (3, 554)	57 (21, 89)	0.004[a]
Butanoic acid, 3-methyl	7 (0, 48)	141 (69, 406)	28 (1, 962)	68 (7, 590)	0.035[a]
Phenol	9 (3, 35)	68 (21, 188)	21 (5, 180)	13 (5, 22)	0.021[a]

Median and upper and lower quartile values are shown; Kruskal–Wallis 1-way analysis of variance examined differences between groups.
[a]$P < 0.05$ being considered significant.

and 50% Tenax (Markes International Ltd, Llantrisant, United Kingdom).

Laboratory Analysis

AU6 An internal standard solution comprising 50 ng ~~d8-toluene~~ (Supelco Cat No. 48,593) in methanol was added to each tube according to the manufacturer's instructions (Markes International Ltd). Automated thermal desorption–gas chromatography–mass spectrometry analyzed headspace samples. A PerkinElmer system was used for analysis, combining a TurboMass MS 4.1, Autosystem XL GC, and Automatic Thermal Desorption system (ATD 400; AU7 PerkinElmer, Wellesley, MA). The carrier gas was CP-grade helium (BOC Gases, Guildford, United Kingdom) passed through a combined trap for removal of hydrocarbons, oxygen, and water vapor. A wall-coated Zebron ZB624 chromatographic column was used (Phenomenex, Torrance, CA), with dimensions ~~30 × 0.4 × 0.25 mm~~ (internal diameter), the liquid phase comprising a 0.25-μm layer of 6% cyanopropylphenyl and 94% methylpolysiloxane.

Thermal desorption tubes were initially purged for 2 minutes to remove air and water vapor and then desorbed for 5 minutes at 300°C. The automatic thermal desorption valve temperature was set to 180°C, and TD tubes were desorbed onto the secondary cold trap, which was initially maintained at 30°C. Once desorption was complete, the secondary trap was heated to 320°C using the fastest available heating rate and then maintained for 5 minutes, ~~whereas~~ the effluent was transferred to the gas ~~chromatography~~ through a transfer line heated to 210°C. The gas ~~chromatography~~ oven was maintained at 50°C for 4 minutes after injection and then raised at a rate of 10°C/min until reaching 220°C and then held for 9 minutes. A heated line held at 240°C to the mass spectrometer transferred the eluted products where the compounds were subjected to electron ionization. Full scan mode was selected with mass to charge ratios from 33 to 350 m/z with a scan time of 0.3-second and 0.1-second interscan delay to produce a total ion chromatogram.

Data Analysis

Compound identification was achieved using Automated Mass Spectral Deconvolution and Identification (AMDIS version 2.62) software and the National Institute of Standards and Technology mass spectral library. Quantification was achieved by comparing the area of each compound peak with the peak area associated with the known amount of ~~d8-toluene~~.

In any given fecal headspace sample, AMDIS would identify from (approximately) 100 to 300 different compounds and it was therefore found necessary to select a subset of those we observed to render statistical analysis tractable. Three approaches were followed to provide a list of what we have termed "candidate compounds." The list comprised first compounds that appeared to be most abundant from inspection of the results obtained using AMDIS; second, compounds that appeared to discriminate between patient groups by visual inspection of a subset of pre-
F1 treatment sample chromatograms (Fig. 1 for an example); and third, compounds selected on the basis of published work. For the latter approach, an initial generic list was made including short-chain fatty acids (SCFAs) and their derivatives, phenolic compounds and indole, and sulphides.[30–32] This list was then refined according to publications dealing more explicitly with VOC profiles in GI disease.[25,33,34] A final list of 13 compounds was obtained in this way.

The frequency distributions for all compounds were found to be highly skewed with a proportion of nondetects; therefore, a nonparametric statistical approach was adopted that is described in detail in the Results section.

ETHICAL CONSIDERATIONS

The Leeds West Local Ethical Committee granted ethical permission for this study (Ref. 07/Q1205/39).

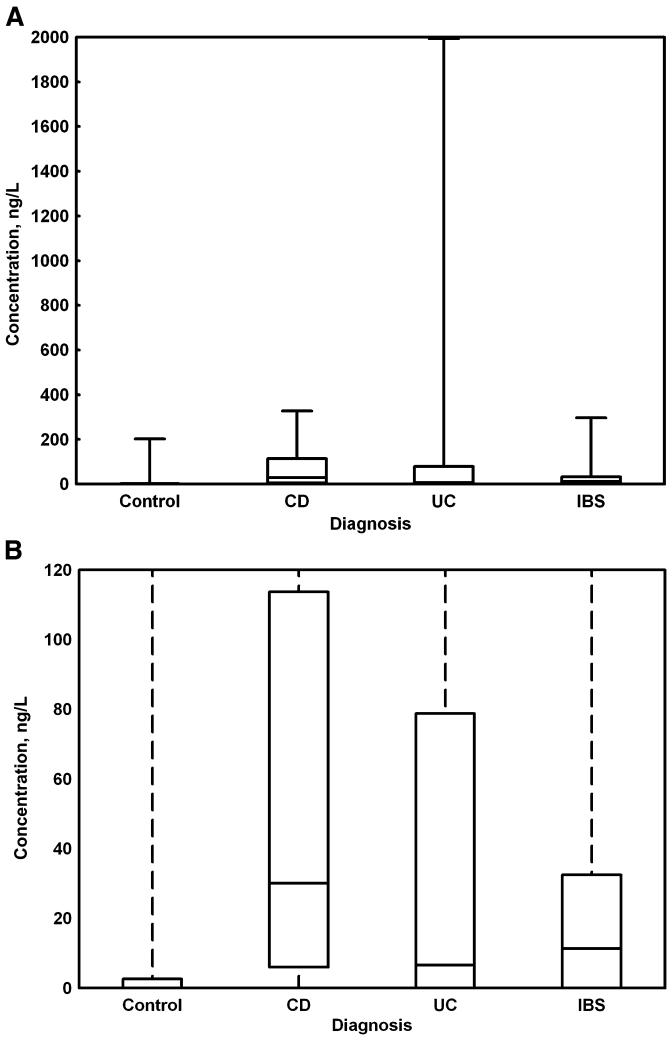

FIGURE 2. Box-and-whisker plots of the concentration of butanoic acid methyl ester in patients with GI disease and healthy controls, before prescribed treatment. The boxes indicate the lower and upper quartiles, the transverse bars the median, and the whiskers indicate the range. A, Whole data range. B, Expanded vertical scale to show relative locations of medians and quartiles.

RESULTS

Table 2 lists the 13 candidate compounds according to their method of selection and typical gas chromatography retention times. Statistical analysis was carried out using STATISTICA version 10 (Statsoft, Inc, Tulsa, OK).

Concentrations of each candidate compound from the control group and volunteers with CD, UC, and IBS obtained before treatment were compared using a Kruskal–Wallis 1-way analysis of variance. The comparison of the control and disease groups before treatment is shown in Table 3.

Because of the highly skewed frequency distribution observed for all compounds, median and upper and lower quartile values are listed. Statistically significant differences were observed for 8 of the 13 candidate compounds with at least 1 compound found to be significant from each method of selection. Where significant differences did occur, they were always associated with elevated concentrations of the relevant compound in the disease groups relative to the controls. The largest elevations were associated with the CD group.

Despite the statistically significant differences between groups, there was considerable overlap in the values of the measured concentrations and all compounds exhibited a wide dynamic range in all groups including the controls. This is illustrated in Figure 2 for a representative compound, butanoic acid methyl ester. The wide dynamic range of the observed concentrations necessitated plotting the data on 2 different scales to allow both the whole range to be shown and the relative locations of medians and quartiles.

Table 4 shows the comparison of the control and disease groups following appropriate treatment regimens as described in the Methods section. Generally, a striking reduction in the concentrations of the candidate compounds was observed in the disease groups, and this was reflected in there being fewer statistically significant differences among all groups (controls and patients) assessed using the Kruskal–Wallis test. The only significant difference persisting was in propanoic acid ethyl ester ($P = 0.003$), and even here, the observed concentrations in the disease groups were markedly lower than those observed pre treatment. The effect of treatment on a representative compound, butanoic methyl ester, is illustrated in Figure 3. This has been plotted to the same scale as Figure 2 to facilitate comparison of pretreatment and posttreatment data. As before, 2 plots are provided to take account of the wide dynamic range of the data.

Where statistically significant differences were observed, post hoc tests were carried out using the z statistic described by Siegel and Castellan[35] to establish the existence of significant differences between each pair of subject groups. The results of this analysis are presented in Table 5, which shows the value of the z statistic and associated P value for each pairwise comparison. It is notable that 1 pairwise comparison was found to be statistically significant for each compound; this was invariably between the control group and CD patients.

One limitation of this analysis is that it was not possible to obtain both pretreatment and posttreatment samples from all volunteers. Consequently, the data could be considered to have been obtained in groups that are at least partly independent and

TABLE 4. Concentrations of VOCs in Fecal Headspace From Healthy Controls and Volunteers Diagnosed With CD, UC, and IBS Following Prescribed Treatment

	VOC Concentration, ng/L				
	Median (Lower Quartile, Upper Quartile)				
Compound	Control	CD	UC	IBS	P
Acetone	142 (21, 336)	80 (50, 104)	59 (38, 123)	100 (73, 146)	0.498
Propanoic acid	6 (0, 55)	14 (0, 84)	62 (0, 220)	175 (0, 529)	0.432
Butanoic acid	33 (3202)	53 (0, 104)	203 (7, 778)	111 (5, 1445)	0.348
1-Propanol	13 (0, 33)	146 (6, 661)	10 (5, 101)	20 (6, 62)	0.199
Propanoic acid, ethyl ester	0 (0, 0)	2 (0, 15)	0 (0, 0)	0 (0, 0)	0.003[a]
Butanoic acid, methyl ester	0 (0, 3)	1 (0, 5)	0 (0, 1)	4 (0, 21)	0.058
Butanoic acid, ethyl ester	0 (0, 1)	2 (0, 21)	0 (0, 0)	0 (0, 0)	0.058
P-cresol	122 (20, 433)	505 (213, 1234)	477 (80, 1309)	301 (112, 854)	0.203
Indole	9 (3, 34)	32 (0, 128)	13 (5, 142)	39 (3, 110)	0.723
Dimethyl disulphide	30 (4, 71)	77 (20, 149)	43 (23, 133)	130 (20, 190)	0.408
1-Butanol	9 (0, 39)	86 (6, 204)	7 (2, 151)	39 (0, 265)	0.496
Butanoic acid, 3-methyl	7 (0, 48)	6 (0, 45)	2 (0, 243)	129 (2, 552)	0.171
Phenol	9 (3, 35)	31 (16, 181)	14 (5, 21)	11 (5, 28)	0.092

Median and upper and lower quartile values are shown; Kruskal–Wallis 1-way analysis of variance examined differences between groups.
[a]$P < 0.05$ being considered significant.

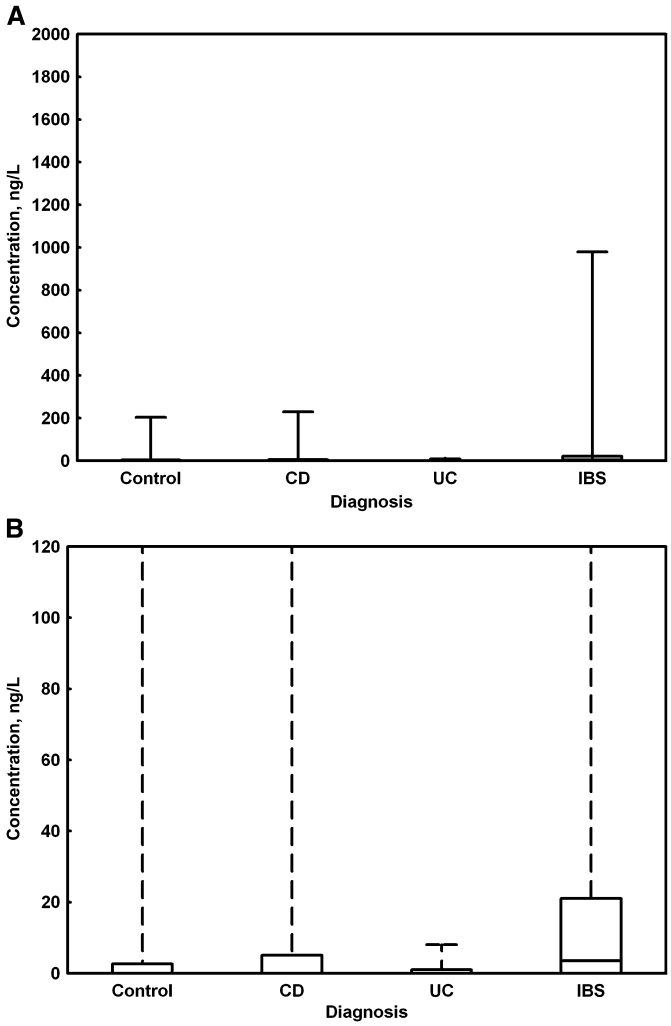

FIGURE 3. Box-and-whisker plots of the concentration of butanoic acid methyl ester in patients with GI disease and healthy controls, following prescribed treatment. The boxes indicate the lower and upper quartiles, the transverse bars indicate the median, and the whiskers indicate the range. A, Whole data range. B, Expanded vertical scale to show relative locations of medians and quartiles. Plotted to the same scale as Figure 2 to facilitate comparison with pretreatment results.

hence to have arisen by chance. A further analysis was therefore carried out using the Wilcoxon signed-rank test to compare VOC concentrations in pretreatment and posttreatment samples for those cases where paired data were available. The numbers of paired samples available were 17, 7, and 16 for CD, UC, and IBS groups, respectively. The results are shown in Table 6. It will be noted that the number of paired samples ("n" in Table 6) differs by compound within each volunteer group. This arose because of the difficulty in obtaining positive identification of compounds when present at very low concentration, where the signal-to-noise ratio was relatively poor. In some cases, a peak was discernible at the appropriate retention time for a given compound but positive identification could be made neither by AMDIS nor by visual inspection of the mass spectrum. In these cases, data were recorded as missing; it being considered less likely to bias the results than recording a nondetect or the concentration calculated on the dubious peak.

The results are broadly consistent with the unpaired analyses (Tables 3–5). Of the 13 candidate compounds, 7 were found to be significantly reduced following treatment in the CD group, with no significant differences observed in either UC or IBS. Of these 7, 6 were also found to be statistically significant in the unpaired analysis of pretreatment data (Table 3). Three compounds, butanoic acid methyl ester, indole, and phenol, were significant in the unpaired analysis but not in the paired analysis. Conversely, a significant reduction in propanoic acid was found in the paired analysis, but this compound was not significantly different between groups in the unpaired analysis. A particularly striking difference was observed in butanoic acid concentration in CD patients from a median of 1075 to 1 ng/L.

DISCUSSION

We have used pumped sampling onto TD tubes to determine the concentrations of a range of VOCs in the headspace of fecal samples from patients with CD, UC, or IBS and also a group of healthy control volunteers. As far as we are aware, this is the first report in which quantification of VOCs in fecal headspace has been attempted and also the first to examine the effects of prescribed treatment. Although a fecal headspace sample can yield more than a hundred different volatile species, we selected a relatively small number (13, decided empirically) of candidate compounds to render statistical analysis tractable. Selection was carried out, with no a priori hypothesis, on the basis of relative abundance, indications from previous studies, and inspection of a small subset of our own data. This was a highly empirical method but proved to be effective. We also considered using a multivariate approach to analyze these data, but this was precluded for a variety of reasons, particularly the proportion of missing data and the highly skewed frequency distribution of all variables, which together with the presence of a number of nondetects made it impossible to normalize the data by transformation. The compounds selected can be considered as falling into 3 broad categories: SCFAs and their derivatives (propanoic and butanoic acids and their corresponding alcohols, esters, and branched derivatives), products of protein metabolism (indole, phenol, and para-cresol), and other common bacterial metabolites (acetone and dimethyl disulphide).

The concentrations of propanoic and butanoic acids were highest in the CD group, although statistically significant only for butanoic acid. SCFAs originate largely from bacterial fermentation of polysaccharides and dietary fiber, although they can also be produced by the metabolism of polypeptides from dietary or endogenous sources such as bleeding, mucins, or sloughed cells.[31] SCFAs are considered to have beneficial effects on the human colon. They can act as a source of energy, affect colonic mucosal growth, promote the absorption of sodium and water, and mitigate against diarrhea. Butanoic acid is thought to have a protective

TABLE 5. Pairwise Comparisons Between Groups Examined Using the ~~Z~~ Statistic of Siegel and Castellan

	Post Hoc Comparisons Between Groups (~~Z~~ Value, *P*)					
Compound	Control Versus CD	Control Versus UC	Control Versus IBS	CD Versus UC	CD V ~~ersus~~ IBS	UC Versus IBS
Pretreatment data						
Butanoic acid	2.71	1.27	2.14	1.37	0.50	0.85
	0.041[a]	1.000	0.194	1.000	1.000	1.000
1-Propanol	3.56	1.85	0.85	1.52	2.39	0.89
	0.002[a]	0.387	1.000	0.767	0.102	1.000
Propanoic acid, ethyl ester	3.93	2.39	1.72	1.38	2.16	0.70
	0.001[a]	0.102	0.511	1.000	0.183	1.000
Butanoic acid, methyl ester	3.16	1.83	1.73	1.03	1.42	0.25
	0.009[a]	0.403	0.506	1.000	0.936	1.000
Butanoic acid, ethyl ester	3.26	2.22	1.46	0.63	1.90	0.98
	0.007[a]	0.160	0.858	1.000	0.345	1.000
Indole	4.05	1.82	1.80	2.18	2.13	0.01
	0.000[a]	0.412	0.429	0.178	0.202	1.000
1-Butanol	3.60	1.58	1.55	1.86	2.08	0.11
	0.002[a]	0.683	0.734	0.378	0.224	1.000
Butanoic acid, 3-methyl	2.88	1.39	2.00	1.21	0.82	0.45
	0.024[a]	0.990	0.273	1.000	1.000	1.000
Phenol	2.68	1.38	0.21	1.11	2.55	1.21
	0.044[a]	1.000	1.000	1.000	0.064	1.000
Posttreatment data						
Propanoic acid, ethyl ester	2.49	0.488	0.753	1.71	1.92	0.144
	0.076[a]	1.000	1.000	0.518	0.326	1.000

These analyses were completed post hoc for those compounds for which the initial Kruskal–Wallis test produced a statistically significant result.
[a]~~Differences were considered statistically significant.~~

effect against colonic cancer, but the evidence for this is equivocal.[36] Incubation of fresh stool with labeled butanoic acid has demonstrated the production of a range of esters and also 1-butanol,[25] and it is likely that homologous series of metabolites are produced from other SCFAs. Much less is known about the physiological effects of these derivatives, although it has been suggested that 1-propanol may be damaging to the gut.[25] There is also evidence that ethyl esters of fatty acids may exert a toxic effect by inducing mitochondrial dysfunction.[37,38] Although these findings pertain to derivatives of long-chain fatty acids, they are nonetheless striking in view of the high concentration of fatty acid esters observed, especially in the case of CD.

In contrast to SFCAs, the products of amino acid fermentation, indole, phenol, and p-cresol are generally considered to be toxic to the gut. We detected statistically significant elevations in the concentration of indole and phenol in CD patients compared with controls. Concentrations were also higher in the UC and irritable bowel disease groups than in the controls but were not found to be significant in post hoc tests between pairs of groups (Table 5). Para-cresol was widely detected in fecal headspace samples with the highest concentrations observed in CD; however, there was no statistically significant difference between groups.

No significant differences were found in acetone concentration. This might be expected because acetone was selected as a candidate compound on the basis of high relative abundance and is a product of lipolysis by many organisms including bacteria. Similarly, dimethyl disulphide is produced as a result of the bacterial metabolism of sulfur-containing compounds (e.g., proteins containing disulphide bonds) and is one of the compounds that contribute to the characteristic odor of feces.[32] The absence of any significant changes in these general markers of bacterial metabolism suggests that our findings reflect changes in the balance of the gut microbiota rather than a change in the overall number of bacteria or increase in metabolic activity. This also provides some reassurance that our results are not significantly affected by variations in sample consistency or water content.

The use of gas chromatography as a method for identification of bacteria is by no means new. As early as 1967, Lewis et al[39] observed significant differences in SCFA production between different species of *Clostridium* and subsequent studies

extended this to other clinically relevant bacteria and the direct analysis of clinical material.[40–42] It is only more recently, however, that the relatively easy availability of automated mass spectrometry techniques has allowed this approach to be fully exploited. To date, there have appeared only a limited number of reports of volatile profiling in fecal samples. Garner et al[25] used solid-phase microextraction and gas chromatography–mass spectrometry to demonstrate the diagnostic potential of fecal VOCs in UC and *C. difficile* and *C. jejuni* infection. It is difficult to compare these results with the current data because they are expressed in terms of presence/absence scores for each of a very wide range of compounds that were then analyzed using a multivariate approach. Although highly effective in separating the groups in their study, this method makes it difficult to ascribe the observed differences to particular compounds. Our use of pumped TD tubes has enabled us to estimate the quantities of VOCs produced by fecal samples, and in fact, all those in the candidate compound list were widely observed in samples from all the groups in our study. De Preter et al[34] have developed a method employing purge-and-trap sampling that allows quantification of fecal headspace VOCs, although as far as we are aware at the present time, only data from a group of healthy volunteers have been reported.

We found pronounced differences in the median concentrations of a number of VOCs in the headspace of feces of patients with GI disease compared with healthy control volunteers, which suggests that this method has potential diagnostic value. Despite the large magnitude of the differences, however, the data occupy a wide dynamic range and there remains considerable overlap in the values observed in each group, as illustrated in Figure 2 for butanoic acid methyl ester. It therefore seems likely that a panel comprising several marker compounds would be required to provide sufficient discrimination for the diagnosis of individual cases. Of the compounds determined in this study, our results indicate that the best discrimination might be provided by propanoic acid ethyl ester, butanoic acid methyl ester, 1-propanol, 1-butanol, and possibly also indole.

A potential limitation on the practical employment of this method is that statistically significant differences in VOC concentration were found only between the CD and control groups (Table 5), which suggests that differential diagnosis of Crohn's disease, UC, and IBS might require some refinement. Nonetheless, there persist large relative differences in the median concentrations of some compounds between the disease groups, notably propanoic acid, 1-propanol, and butanoic acid ethyl ester (Table 3). This area merits further investigation, possibly using modified sampling and analytical protocols to minimize methodological variability, and the use of multivariate data analysis techniques to cope with the high dimensionality of the resultant data sets.

Our results clearly show significant differences in VOC concentration between the control and disease groups, which reflect changes in the gut microbiota found in these conditions. It is however impossible in the current state of knowledge to relate the two in detail. It is known that in IBD, Actinobacteria and Firmicutes are relatively inactive or dormant, whereas there is an expansion of the Proteobacteria, especially *Escherichia coli*, *Campylobacter concisus*, and *Helicobacter spp.*[5,6] However, changes in

TABLE 6. Comparison of Paired Pretreatment and Posttreatment Fecal Headspace VOC Concentrations Using the Wilcoxon Signed-Rank Test

Paired Comparisons Between Pretreatment and Posttreatment VOC Concentrations			
	Median Pretreatment, Median Posttreatment (n, *P*)		
Compound	CD	UC	IBS
Acetone	64–83 (16, 0.352)	118–65 (7, 0.735)	84–109 (14, 0.300)
AU9 Propanoic acid	125–18 (14, 0.048[a])	0–1 (5, 0.345)	21–286 (13, 0.064)
Butanoic acid	1074–1 (15, 0.002[a])	4–10 (7, 0.398)	362–576 (13, 0.807)
1-Propanol	281–123 (15, 0.041[a])	0–9 (7, 1.000)	46–37 (10, 0.386)
Propanoic acid, ethyl ester	49–1 (13, 0.002[a])	—	0–0 (6, 0.249)
Butanoic acid, methyl ester	14–0 (12, 0.084)	0–1 (2, 0.655)	18–14 (12, 1.000)
Butanoic acid, ethyl ester	68–1 (15, 0.011[a])	0–0 (2, 0.655)	2–0 (8, 0.123)
P-cresol	711–530 (15, 0.820)	29–85 (7, 0.612)	426–424 (11, 0.534)
Indole	127–44 (14, 0.177)	25–13 (7, 0.499)	41–56 (12, 0.754)
Dimethyl disulphide	67–64 (12, 0.388)	78–43 (6, 0.388)	46–164 (13, 0.753)
1-Butanol	142–86 (13, 0.011[a])	6–12 (6, 0.917)	58–43 (14, 0.551)
Butanoic acid, 3-methyl	147–28 (14, 0.026[a])	5–123 (6, 0.753)	104–276 (14, 0.638)
Phenol	84–24 (13, 0.133)	94–9 (6, 0.345)	12–9 (14, 0.975)

Em dash indicates insufficient paired data to permit comparison.

the composition of the bacterial population do not necessarily reflect overall metabolic activity, and it has in fact been suggested that species at lowest abundance might in fact be metabolically the most active.[43] An added complication of uncertain effect is the presence of relatively small numbers of viruses and fungi. It therefore seems unlikely that changes in abundance of individual species will relate directly to the observed changes in VOC profile. However, our results are consistent with the results of earlier studies by Garner et al,[25] who demonstrated the production of SCFAs and their derivatives from fecal cultures obtained from IBD patients, and by van Nuenen et al,[33] who found increased production of short-chain and branched-chain fatty acids by feces from IBD patients compared with healthy individuals when cultured in an in vitro model of the large intestine.

These differences are largely normalized following treatment, when the VOC profile more closely resembles that found in the controls (Table 4), indicating reestablishment of a relatively normal microbiota. Following treatment, the overall pattern was of large reductions in the concentration of VOCs, with particularly striking changes in the CD group for butanoic acid (1037–54 ng/L), propanoic acid ethyl ester (26–2 ng/L), and butanoic acid ethyl ester (30–1 ng/L). However, some compounds did exhibit increased concentrations following treatment, almost entirely confined to the UC and IBS groups. Although none of these differences were statistically significant, this observation merits further study.

Analysis of paired data produced broadly similar results with statistically significant reductions in a range of VOCs observed in the CD patients but with some sizable nonsignificant increases in some compounds following treatment: The elevation in propanoic acid from 21 to 286 ng/L after treatment for IBS is particularly notable. These data suggest that fecal VOC analysis might provide a suitable means for monitoring disease process and efficacy of treatment in patients with established primary diagnoses. For this to be viable, concentrations of the relevant compounds would need to be measured and assessed on an individual basis. Clearly, however, the sampling, analysis, and interpretation of such data would need significant development to be useful for clinical practice; however, it is envisaged that with developments in gas sensing, it is feasible that a small number of targeted compounds could be readily measured in the headspace of freshly voided feces in a purpose built device. This would greatly reduce the complexity of instruments and algorithms for their detection and demonstrates the value in targeting a small number of compounds for analysis.

In summary, pumped sampling onto TD tubes is an effective method for the quantification of fecal VOCs. Marked differences in VOC concentrations were observed between healthy controls and patients with GI disease, indicating the potential for a rapid noninvasive diagnostic method. Of the compounds determined, the ester and alcohol derivates of SCFA show most promise for further development. Normalization of the fecal VOC profile following treatment suggests that this approach could also be useful in monitoring progress of disease and the effectiveness of therapy.

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