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Investigation of Volatile Organic Compounds Emitted from Faeces for the Diagnosis of Giardiasis

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ABSTRACT

Background: Giardiasis is a common intestinal infection caused by the flagellated intestinal protozoan *Giardia duodenalis.* Several methods are available for the laboratory diagnosis of Giardia, ranging from the microscopic identification of the parasite trophozoite and cyst stages, to immunodiagnosis and PCR. *Giardia* has unique metabolic pathways resulting from its lack of mitochondria, making it an ideal target for volatile organic compound (VOC) profiling.
Aim: To characterise the VOC profile of stool infected with *Giardia* to detect differences from those found in samples of diarrhoea without *Giardia* or other infections.
Method: Stool was obtained from patients with confirmed *Giardia* infection and controls with diarrhoea but no identifiable infection. Faecal headspace gas extraction and gas chromatography-mass spectrometry were used to extract and identify VOCs.
Results: More than 100 VOCs were identified when control and *Giardia* groups were combined, of which 24 showed significant differences between the two groups ($p<0.05$). Three VOCs had a significantly greater prevalence amongst *Giardia* cases ($p<0.0001$) and 9 VOCs showed a significant difference in terms of abundance ($p<0.05$). AUROC analysis demonstrated a value of 0.902.
Conclusion: There is a significant difference in the VOC profile of stool from subjects infected with *Giardia* spp, when compared with non-infected controls. These findings can be explained by the unique metabolism of *Giardia*.

Key words: giardiasis – volatile organic compounds – diarrhoea.

Abbreviations: AMDIS: Automated Mass Spectral Deconvolution and Identification System; AUROC: area under receiver operating characteristic; EIA: Enzyme immunoassay; GCMS: Gas Chromatography Mass Spectrometry; IBS: irritable bowel syndrome; NIST: National Institute of Standards and Technology; PCA: principle component analysis; PCR: polymerase chain reaction; PLS-DA: partial least squared discriminant analysis; SPME: Solid phase microextraction; TIC: total ion current; VOC: volatile organic compound.

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INTRODUCTION

Giardiasis is a common worldwide intestinal infection [1], caused by the flagellated intestinal protozoan *Giardia duodenalis* (syn. *Lamblia intestinalis*). *Giardia* is transmitted via the faecal-oral route or via contaminated water or food. Giardiasis is accompanied by diarrhoea, severe abdominal pain and flatulence and it may, therefore, mimic irritable bowel syndrome (IBS) [2]. Patients with giardiasis are reported to expel a foul gas, as an eructation or as flatus, with a distinctive smell. This has been attributed to the anaerobic metabolism of cysteine, by *G. duodenalis*, to produce hydrogen sulphide gas [3]. *Giardia* is a highly diverse parasite with seven genetic assemblages (A-G), or cryptic species, infecting different hosts. Polymerase chain reaction (PCR) amplification of appropriate genes, followed by DNA sequencing, can be used to distinguish the assemblages. Humans are infectable with the assemblages A and B, the latter being predominant. Differences in the disease outcome, that are apparently related to the *Giardia* assemblage causing infection have been reported, but the results are inconsistent [4].

The life cycle of *Giardia* consists of an environmentally resistant infectious cyst form and a motile trophozoite. The cyst is oval, typically 7-10μm wide, containing 4 nuclei with a rigid outer wall that protects it from extreme conditions, including temperature and chlorination. Ingestion of as few as 10 cysts can lead to infection. Excystation occurs after ingestion once the cyst comes into contact with gastric acid and intestinal proteases. In the duodenum, a single cyst gives rise to 4 trophozoites, each containing 2 nuclei and no mitochondria. The trophozoites adhere to the intestinal enterocytes and cause diarrhoea. Encystation results from exposure of the trophozoite to alkaline conditions or bile salts. Cysts are excreted in the stool.

Cysts have a metabolic rate that is 10-20% of that of trophozoites. Their metabolism is stimulated by ethanol [5]. The trophozoites are microaerophilic and, because they lack mitochondria, they rely on cytochrome-mediated oxidative phosphorylation. They can perform aerobic and anaerobic metabolism depending on environmental oxygen concentration, although they predominately rely on fermentation which is carried out even when oxygen is present [6].

There are several methods for the laboratory diagnosis of *Giardia*, ranging from the identification of the parasite trophozoite and cyst stages by microscopy of stools, to more sensitive biochemical and molecular approaches such as immunodiagnosis and PCR [7]. Commercially available enzyme immunoassay (EIA) tests have sensitivities of >90% and specificity of up to 100% [8] which greatly out-performs microscopy of a single stool sample [9]. In Lancashire, a significant increase in the rate of giardiasis was reported after the introduction of an EIA test for diagnosis [10]. However, the diagnosis of *Giardia* in most laboratories still relies on the less sensitive microscopic methods, and new approaches that are simple, cost-effective and which allow a high-throughput of specimens are needed to complement or replace microscopy [7].

We have previously investigated the volatile organic compounds (VOCs) emitted from faeces [11]. Faecal VOCs are produced by the intestinal microbiota and the host. Some VOCs are derived in part by metabolism of food, but they also arise from the metabolism of the microbiota [12]. Importantly, it has been shown that VOCs change in the presence of intestinal disease including infection with *Campylobacter* spp., *Clostridium difficile, Vibrio cholera* as well as inflammatory bowel disease and diarrhoea-predominant IBS [2, 11, 13].

The aim of this study was to determine the pattern of VOCs from stools of patients with *Giardia* infection and compare it with stools of patients with diarrhoea secondary to other conditions. The VOCs pattern of patients infected with *Giardia* assemblage A and those with assemblage B were also compared.

METHODS

Patient sample collection

Faecal specimens from 16 patients with giardiasis were provided by the Royal Preston Hospital (Central Lancashire). *Giardia* infection was diagnosed using a faecal EIA method (*GIARDIA/CRYPTOSPORIDIUM CHEK*®, Techlab, Blacksburg, VA, USA) followed by a *Giardia*-specific immunochromatographic assay (RIDA®QUICK *Giardia*, R-Biopharm, Darmstadt, Germany) for confirmation. These specimens were also tested for six other common intestinal pathogens (*Cryptosporidium, Salmonella, Shigella, Campylobacter, Escherichia coli* 0157 and *Vibrio* spp.) and were found to be negative.

The 17 control samples were obtained from patients with diarrhoea from Bristol Royal Infirmary. They were negative for *Cryptosporidium, Salmonella, Shigella, Campylobacter, Escherichia coli* 0157 and *Vibrio* spp. and the presence of either *Giardia* or *Cryptosporidium* was excluded using the same EIA method described above.

Hard plastic ("Universal") containers were used to collect samples, which were subsequently processed in two laboratories. The storage containers used in the Preston laboratory are not known; the Bristol laboratory used the Universal containers for initial characterization, followed by storage in glass vials. We accept that this is not the ideal collection process as it is open to plasticiser contamination. In the VOC laboratory, 500mg aliquots of faeces were stored in 10ml glass vials (Supelco, Poole, UK) and then frozen at -20°C until analysis.

Faecal headspace gas extraction

Samples were heated to 60˚C for 30 minutes in a water bath to thaw the sample and facilitate VOC release [14]. VOCs were extracted from the headspace gas using a 85µm CAR/PDMS solid phase microextraction (SPME) fibre. The fibre was exposed for 20 minutes during which time the sample remained heated, then it was transferred to the GCMS for desorption. An internal standard was not used, as we were unsure of the VOCs that would be identified and how it could potentially saturate the fibre, thus affecting the detection of other VOCs.

Gas Chromatography-Mass Spectrometry

A PerkinElmer Clarus 500 GC/MS quadrupole bench-top system gas chromatography-mass spectrometry (GCMS) was used to separate and detect the VOCs. The GC column used was a Zebron ZB-624 with inner diameter 0.25 mm, length 60 m, film thickness 1.4 µm (Phenomenex, Macclesfield, UK). The carrier gas used was helium of 99.996% purity (BOC, Sheffield, UK). The SPME fibres used were DVB-CAR-PDMS 50/30 µm (1 cm) (Sigma-Aldrich, Dorset, UK). A splitless injection method was used. The fibre desorption conditions were 5 minutes at 220°C. The initial temperature of the GC oven was set at 40°C and held for 1 minute before increasing to 220°C at a rate of 5°C/min and held for 4 min with a total run time of 41 min. A solvent delay was set for the first 6 min and the MS was operated in electron impact ionization EI+ mode, scanning from ion mass fragments 10 to 300 m/z with an interscan delay of 0.1 sec and a resolution of 1000 at FWHM (Full Width at Half Maximum). The helium gas flow rate was set at 1 ml/min [14].

Identification and comparison of volatiles

Mass spectral data were analysed using Automated Mass Spectral Deconvolution and Identification System (AMDIS) (AMDIS-version 2.71, 2012) and the National Institute of Standards and Technology (NIST) (version 2.0, 2011) database. Three components were used within NIST to identify the compounds, these were a Match of >800, the probability and a head to tail comparison of the fragments. A compound was considered to be present when it satisfied these 3 criteria. This process provides a relative ion abundance, therefore no units of ion abundance are available. Further processing and alignment of data was performed using the Metab package in R [15]. Lists of compounds emitted from samples from patients and controls were made. Log transformation of the abundance data was also performed in order to reduce the skew that was present. Compounds that were absent were assigned a NA label, to avoid a log (0) value. A VOC that is present in less than 20% of all samples cannot be considered to be a biomarker, therefore compounds that were found in less than 20% of the entire sample cohort were removed from further analysis.

Fisher's exact test was performed to compare the rate of occurrence of each VOC in the two groups, a p value of <0.05 was deemed significant. Principle component analysis (PCA) performed in R and ROC analysis was also performed, using Metaboanalyst (Xia et al 2009). Abundances of the compounds were also compared, on log transformed data and *t* tests. Box plots were drawn to illustrate the data. Coefficient of variance was calculated following log transformation of ion abundance.

Ethics

Full ethical approval was given granted for the collection and analysis of stool samples from the two centres involved. The Bristol part of the study was approved by Wiltshire Research Ethics Committee 06/Q2008/6. The Preston part of the study was approved by Northwest England Research Ethics Committee, 11/NW/0704.

RESULTS

A number of 114 VOCs were found in at least 20% of samples from control and *Giardia* groups. There was no significant difference in the number of VOCs identified between the two groups (56.4 ± 16.3 in Giardia, 58.1 ± 12.6 in controls, p = 0.74) .

The headspace gases were dominated by esters, acids and alcohols. There were few amides, alicyclic compounds, ether compounds or nitrogen containing compounds (Fig. 1), whereas ketones were less prevalent than in earlier studies [11]. There was no significant difference in the classes of compounds found in *Giardia* and control groups.

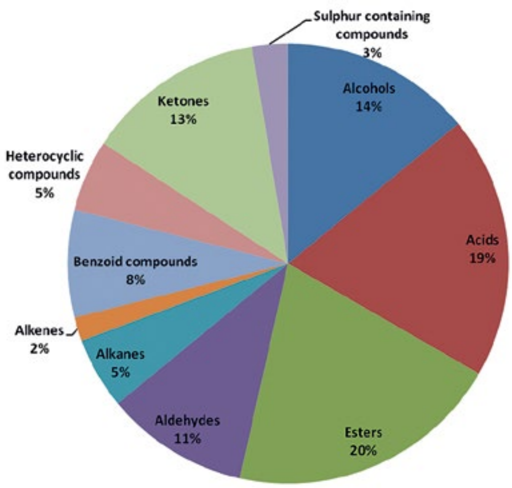

Fig. 1. Pie chart to show the range and classes of compounds found in the headspaces gases of all samples. There was no difference in the class composite between the two study groups, the above chart is representative.

Table I. Compounds with a significantly different prevalence between Giardia and control headspace gas samples

VOC	Present control	Present Giardia	Fishers exact *t* test
2,2,4,4-tetramethyloctane	1	14	<0.0001
Acetic acid	1	12	<0.0001
Heptane, 2,2,4,6,6-pentamethyl	0	10	<0.0001
Cyclopentane	0	7	0.002
2-Pentanone	8	0	0.002
Decane, 2,6,6-trimethyl	1	8	0.006
1-propanol	16	8	0.006
Furan, 3-methyl-	7	0	0.007
Benzene, 1,3-bis(1,1-dimethylethyl)	0	6	0.007
Ethylalcohol	10	2	0.01
Pyrazine, 2,5-dimethyl	6	13	0.013
Pentanoic acid	6	13	0.013
Octanal	13	5	0.014
Pyrazine, 3-ethyl-2,5-dimethyl	2	8	0.025
Propanoic acid	7	13	0.032
Pentanal	4	10	0.036
2(3H)-Furanone, dihydro-5-methyl-	1	6	0.039
2-Hydroxy-3-pentanone	0	4	0.044
Pentane, 2,2,3,3-tetramethyl	0	4	0.044
5-Ethylcyclopent-1-enecarboxaldehyde	0	4	0.044
2-octenal, (E)	0	4	0.044
Propanoic acid, 2-methyl-, ethyl ester	5	0	0.044
Xylene	5	0	0.044
Cyclohexene,1-methyl-4-(1-methylethylidene)-	5	0	0.044

Of the 114 compounds found in at least 20% of patients in either group, 24 compounds achieved a significance of p <0.05 (Table I).

The compounds with the lowest probability, inferred by their respective p value, were 2,2,4,4-tetramethyloctane, acetic acid and 2,2,4,6,6-pentamethylheptane (p <0.0001). 2,2,4,4-tetramethyloctane and acetic acid were both absent in 16 controls, whilst being present in 14 and 12 *Giardia* samples respectively. 2-pentanone was absent in all 16 *Giardia* samples, whilst being present in 8 of the 17 controls. A chromatogram acetic acid was obtained using a pure reference solution of acetic acid was overlaid on a *Giardia* samples and the mass fragment were compared to confirm its identity. There may have been co-elution of acetic acid with one or more VOCs, as suggested by the chromatogram. However, we expect AMDIS to be able to de-convolute this peak and we manually checked its identification by comparing mass spectra and a subsequent pure reference solution (Fig. 2).

Seven compounds were absent in all control samples, providing a negative predictive element: these were

2,2,4,6,6-pentamethylheptane, cyclopentone,1,3-bis(1,1-dimethylethyl)benzene, 2-hydroxy-3-pentanone, pentane, 2,2,3,3-tetramethyl, 5-ethylcyclopent-1-enecarboxaldehyde and 2-octenal, (E).

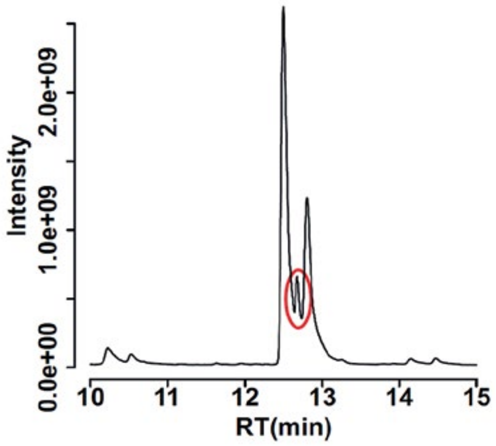

Fig. 2. Representative chromatogram generated from total ion current (TIC) chromatogram. It is representative of the area on the AMDIS chromatogram that the acetic acid peaks were found in the Giardia samples. The red circle highlights the peak identified as acetic acid.

When considering the abundances of the VOCs, there were 9 significantly different compounds ($p<0.05$) (Table II). There was both up- and downregulation of the VOCs in the *Giardia* samples.

Acetone and 2-butanone were the two significantly different compounds ($p <0.001$): both were negatively associated with *Giardia* samples (Fig. 3).

Within the *Giardia* samples, the compounds which showed the greatest abundances were 2-methylphenol and 4-methylphenol and those with the lowest abundances were n-amyl isovalerate and nonanal. There was no significant difference in these four compounds between the two groups.

Table II. The compounds achieving a $p<0.05$ in terms of relative abundance, including Student's t test value. Log transformation of the values has been performed to allow for normalisation.

Volatile organic compound	Mean abundance Control	Mean abundance Giardia	Student *t* test	Coefficient of Variance
Acetone	18.268	16.423	0.0004	5.70
Carbon disulfide	15.372	14.23	0.001	2.40
Dimethyl trisulfide	16.799	14.924	0.007	1.84
Propanoic acid, propyl ester	19.193	16.61	0.011	9.77
Indole	18.597	17.395	0.013	4.15
2-butanone	18.889	17.454	0.02	5.40
4-methyl-phenol	19.355	20.522	0.02	4.84
1-propanol	16.881	15.099	0.04	8.16
Butanoic acid, ethyl ester	18.639	17.055	0.04	8.36

Principle component analysis (PCA), using all the VOCs with a significantly different abundance ($p<0.05$) was performed to illustrate the separation of the two groups (Fig. 4).

ROC analysis was performed using the online tool Metaboanalyst [16]. Using the entire data set univariate analysis was performed (Table III). Further ROC analysis examined the potential as biomarkers of the 9 compounds identified as having significantly different abundances. This used a PLS-DA based multivariate model, again via Metaboanalyst, generating an AUROC of 0.902 (Fig. 5).

Table III. AUROC of the five most significant volatile organic compounds (VOCs) generated using Metaboanalyst software.

VOC	AUROC
2,2,4,4-tetramethyloctane	0.93
1-propanol	0.85
Acetic acid	0.82
2,2,4,6,6-pentamethylheptane	0.81
Acetone	0.80

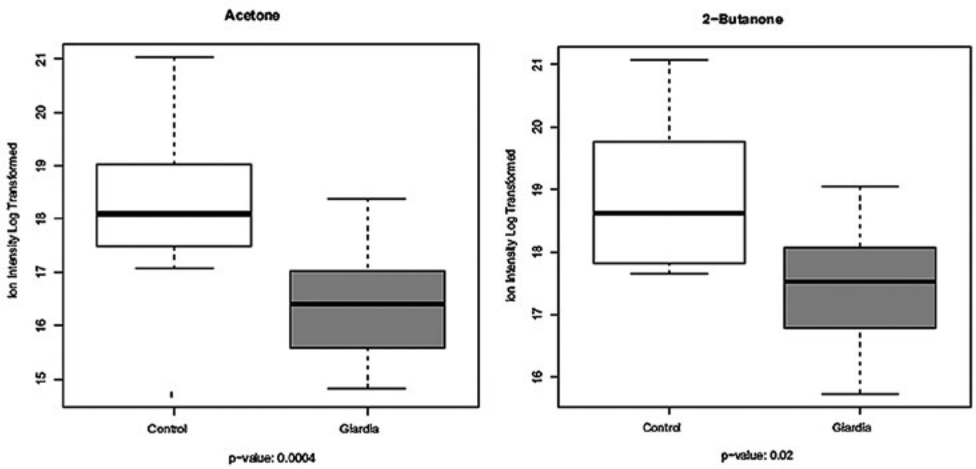

Fig. 3. Box plots showing downregulation of acetone and 2-butanone ($p<0.001$ and 0.02) in *Giardia* samples. The y-axis is a logarithmic scale and is truncated for the range of 15-21, in order to best illustrate the difference.

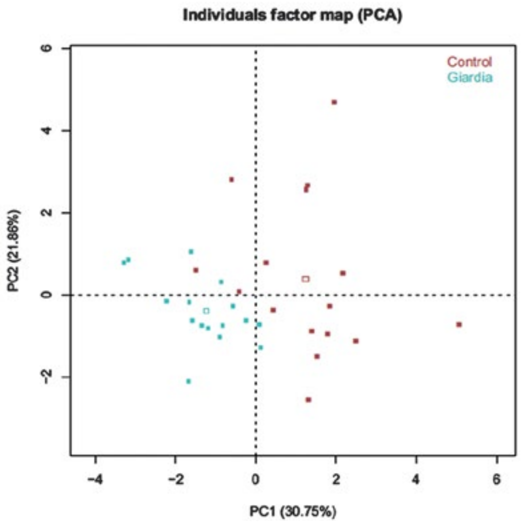

Fig. 4. PCA plot demonstrating the separation of *Giardia* (triangles) and control (circles) samples based on VOCs in the headspace gases. Bold square represents the average control sample, shaded square represents the average Giardia sample.

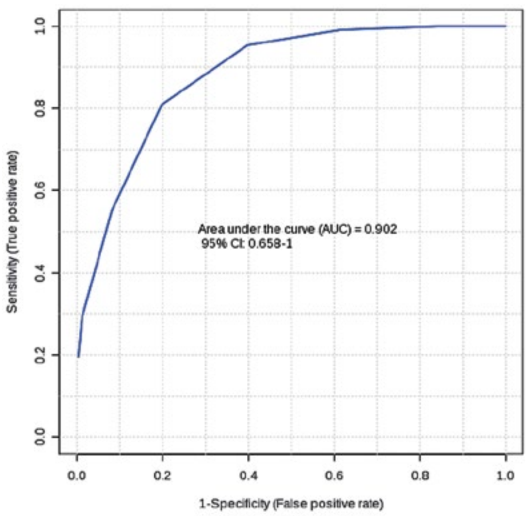

Fig. 5. ROC curve generated using the 9 VOCs with significantly different abundances. Generate using a PLSDA multivariate model.

DISCUSSION

This is the first report of the VOCs emitted from samples of patients with *Giardia*. There are several VOCs that have a significantly different frequency in cases and controls. These changes are likely to originate from the unique metabolism of *Giardia*. The PCA plot figure illustrates the separation between the controls and *Giardia* samples, further emphasising the ability of VOC analysis to differentiate stools from people infected with *Giardia*. It is possible that the differences detected are arising from alterations to the host microbiota as a result of diarrhoea or infection per se, but the differences detected in the VOC profiles have clear links to the unique metabolic pathways of *Giardia*.

Most eukaryotes depend on aerobic metabolism for the production of energy, however *Giardia* does not. The lack of mitochondria and the concomitant cytochrome-mediated oxidative phosphorylation markedly changes the metabolic profile. *Giardia* relies on fermentative metabolism, even when oxygen is present for energy conservation [6]. Glucose is not completely oxidized to CO2 and H2O during metabolism and is instead incompletely catabolized to acetate, ethanol, alanine and CO2 [6]. Carbohydrate metabolism in *Giardia* is profoundly affected by oxygen concentrations. Under strict anaerobic conditions alanine is the major product, but with a very small rise in oxygen levels alanine production changes to ethanol [5]. At an oxygen concentration of >46µM, alanine production is completely inhibited, with acetate and CO2 production predominating. The oxygen concentration seen in the human small bowel is estimated to vary between 0 and 60µM [6], thus causing significant changes to the carbohydrate metabolism seen with *Giardia* and the potential to generate acetate and ethanol.

However, ethanol may be converted to acetic acid and this may explain why acetic acid is often found in giardiasis, but ethanol is not. Ethanol is first oxidised to acetaldehyde by alcohol dehydrogenase. Acetaldehyde is highly unstable and quickly forms free radicals if not transformed. One such transformation is to acetic acid via aldehyde dehydrogenase.

Four alkanes were more frequently found in *Giardia* than in controls. Alkanes are known to be produced by oxidative stress [17] and fatty acid metabolism. *Giardia* has developed elaborate sequences of adaptive mechanisms to maintain oxygen homeostasis and equilibrium [18]. Any deviation from homeostasis, or physiological change in oxygen pressure, is recognized as an exposure to oxidative stress [19]. Oxygen concentration in the small intestine can be up to 60µM, and the conventional enzymes for detoxifying reactive oxygen species e.g. superoxide dismutase, catalase, peroxidase and glutathione reductase are absent in *Giardia* [18]. When exposed to controlled concentrations of dissolved O2 >8 µM, they gradually loose their ability to scavenge O2 [20]. When *Giardia* is exposed to 60µM O2 there is partial collapse of plasma membrane potential and complete loss of O2 uptake [20].

Two ketones, acetone and 2-butanone, were significantly down-regulated in the *Giardia* samples (p <0.001 and 0.02). They have been frequently described in VOC analysis of stool across a variety of conditions and are common in food [11]. Ketogenesis takes place almost exclusively in the mitochondria of hepatocytes in response to low carbohydrate levels. The unique metabolic pathways of *Giardia* degrade ketogenic amino acids to produce acetic acid, potentially explaining the high prevalence of acetic acid and downregulation of acetone and 2-butanone in the *Giardia* samples [21].

The narrow oxygen homeostasis of *Giardia* and the fluctuating levels of oxygen in the small intestine may lead to death related to oxidative stress and release of the alkanes including 2,6,6-trimethyldeance, cyclopentane, 2,2,4,4-tetramethyloctane and 2,2,4,6,6-pentamethylheptane.

Interpretation of the significance of 2,2,4,4-tetramethyloctane and 2,2,4,6,6-pentamethylheptane should be undertaken with caution as they have previously been described as potential contaminates [22]. Plastics were used during this study; all the *Giardia* samples underwent routine testing at Preston and may have been stored in soft plastic. The controls came from Bristol and were stored in hard plastic or glass throughout the study. It is therefore possible that these compounds are contaminates but we feel they should be reported as further work in this area may give clarity to the finding. However, these two compounds do not undermine the findings in relation to acetic acid and acetone summarised in Fig. 5 and Table III.

The VOCs used to generate the PCA were those found to have significantly different abundances, these compounds were then taken forward for AUROC analysis. A value of 0.902 was generated via a multivariate model, further supporting the ability of VOC analysis for the diagnosis of *Giardia* infection.

CONCLUSION

We have described significant changes in the VOCs from faeces of patients infected by *Giardia* spp and the associated metabolic and biological processes leading to their significant prevalence in stool. To explore this further a larger patient population should be studied and severity of the infection could also be assessed and compared. This study has aligned with accepted metabolic pathways unique to *Giardia*. These are initial findings, but they could lead to a point of care diagnosis based on a simple device that recognises specific VOC patterns.

Conflicts of interest: The authors have no issues of conflict to declare.

Authors' contribution: C.P., J.W. and K.L. devised and set up the study. They also were involved with formatting and editing the manuscript. A.B. was responsible for data correlation, statistical analysis, manuscript formation and editing. A.V. and C.M were responsible for performing the laboratory and GCMS work. S.R. and A.M. assisted with statistical analysis and manuscript editing.

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