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Faecal and urine metabolites, but not gut microbiota, may predict response to low FODMAP diet in irritable bowel syndrome

Bridgette Wilson[1,2] | Tokuwa Kanno[3] | Rachael Slater[4] | Megan Rossi[1] | Peter M. Irving[5,6] | Miranda C. Lomer[1,2] | Chris Probert[4] | A. James Mason[3] | Kevin Whelan[1]

[1]Department of Nutritional Sciences, King's College London, London, UK

[2]Department of Nutrition and Dietetics, Guys and St Thomas' NHS Foundation Trust, London, UK

[3]King's College London, Institute of Pharmaceutical Science, London, UK

[4]University of Liverpool, Institute of Systems, Molecular and Integrative Biology, Liverpool, UK

[5]Department of Gastroenterology, Guys and St Thomas' NHS Foundation Trust, London, UK

[6]School of Immunology and Microbial Sciences, King's College London, London, UK

Correspondence
Kevin Whelan, Department of Nutritional Sciences, King's College London, London, UK.
Email: kevin.whelan@kcl.ac.uk

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Summary

Background: The low FODMAP diet (LFD) leads to clinical response in 50%–80% of patients with irritable bowel syndrome (IBS). It is unclear why only some patients respond.

Aims: To determine if differences in baseline faecal microbiota or faecal and urine metabolite profiles may separate clinical responders to the diet from non-responders allowing predictive algorithms to be proposed.

Methods: We recruited adults fulfilling Rome III criteria for IBS to a blinded randomised controlled trial. Patients were randomised to sham diet with a placebo supplement (control) or LFD supplemented with either placebo (LFD) or 1.8 g/d B-galactooligosaccharide (LFD/B-GOS), for 4 weeks. Clinical response was defined as adequate symptom relief at 4 weeks after the intervention (global symptom question). Differences between responders and non-responders in faecal microbiota (FISH, 16S rRNA sequencing) and faecal (gas–liquid chromatography, gas-chromatography mass-spectrometry) and urine (^{1}H NMR) metabolites were analysed.

Results: At 4 weeks, clinical response differed across the 3groups with adequate symptom relief of 30% (7/23) in controls, 50% (11/22) in the LFD group and 67% (16/24) in the LFD/B-GOS group ($p = 0.048$). In the control and the LFD/B-GOS groups, microbiota and metabolites did not separate responders from non-responders. In the LFD group, higher baseline faecal propionate (sensitivity 91%, specificity 89%) and cyclohexanecarboxylic acid esters (sensitivity 80%, specificity 78%), and urine metabolite profile (Q^2 0.296 vs. randomised −0.175) predicted clinical response.

Conclusions: Baseline faecal and urine metabolites may predict response to the LFD.

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Trial registration site and ID: https://www.isrctn.com/ ISRCTN16562415 International Standard Randomised Controlled Trial Number, ISRCTN, 16562415. The research was performed at King's College London, Guy's and St Thomas' NHS Foundation Trust, Barts Health NHS Trust and samples were also analysed at the University of Liverpool and Clasado Biosciences, Reading, UK.

1 | INTRODUCTION

Irritable bowel syndrome is a chronic and debilitating functional gastrointestinal (GI) disorder characterised by abdominal pain and altered bowel habit, affecting 1.5%–4.1% of adults globally.[1]

Altered microbiota and faecal metabolites are part of the pathophysiology of IBS however it is unclear if the changes are a cause or feature of IBS. In a systematic review of 24 studies, Enterobacteriaceae, Lactobacillaceae (family), and Bacteroides (genus) were shown to be higher in IBS than healthy controls, whereas Clostridiales, Faecalibacterium, Bifidobacterium (genus), and *Faecalibacterium prausnitzii* (species) were shown to be lower.[2] Altered microbiota and short-chain fatty acid (SCFA) production in IBS may contribute to low-grade inflammation,[3,4] altered tight junction protein arrangement and increased jejunal humoral immunity described in diarrhoea-predominant IBS (IBS-D).[5,6]

A major approach to dietary management of IBS is the restriction of fermentable oligo-, di-, mono-saccharides and polyols (FODMAPs), termed the low FODMAP diet (LFD),[7] which significantly reduces IBS severity, abdominal pain, overall symptoms and improves bowel habits and quality of life.[8,9] A systematic review including 22 studies identified that the rate of dichotomous clinical response (measured as either >50-point reduction in IBS-SSS score or adequate symptom relief) to the LFD was 61%–69% meaning that 31%–39% of patients that follow the diet will not respond.[8]

The LFD is burdensome to follow, requiring complete dietary change. It may reduce intake of fibre, calcium and iron and decrease diet quality,[10,11] and reduce faecal bifidobacteria concentration and faecal butyrate.[12] Bifidobacteria are considered beneficial for gastrointestinal health due to regulating colonic pH, favourable immune modulation and pathogen exclusion,[13,14] and butyrate contributes to colonocyte epithelial integrity.[13] Therefore, due to the burden for patients in following the LFD and the potential disturbance to the gastrointestinal ecosystem, there would be a significant benefit to both patients and healthcare providers if symptom response to the LFD could be predicted.

A range of biological markers of response to LFD have been proposed. A commercial 'dysbiosis test' has shown conflicting results for individual microbial species in predicting response to the LFD, with one study finding higher Actinobacter and Streptococcus at baseline in responders[15] and another showing that these were both lower in responders at baseline.[16] Volatile organic compounds (VOCs) are intermediaries/endpoints of metabolic pathways that reflect many aspects of colonic metabolism.[17–19] Faecal VOC profiles were demonstrated to differ between responders and non-responders to the LFD,[20] however, individual metabolites were not characterised and specific response-predicting metabolites have yet to be identified.

The current study explores differences in faecal microbiota, and faecal and urine metabolites, between responders and non-responders to the LFD in IBS using data from the previously published 3-arm parallel, placebo-controlled trial. The original paper compared endpoints between the different dietary interventions[12] and these are not described here.

2 | METHODS

2.1 | Study design and participants

The study participants have been described in detail previously.[12] The original study aimed to investigate the effect of the LFD alone or in conjunction with a prebiotic supplement on IBS symptoms and gut microbiota, between-group comparisons were reported previously.[12] Briefly, adult outpatients with Rome III IBS (diarrhoea predominant, mixed, or un-subtyped) were recruited in London, UK via gastroenterology outpatient clinics and randomised to one of three groups: sham diet with placebo supplement (control); LFD with placebo supplement (LFD); or LFD with 1.8 g/d B-GOS supplement (LFD/B-GOS, Clasado Biosciences) for 4-week. Research ethics committee approval was received from the Wales Research Ethics Committee-4 (Reference 15-WA-0119) and all patients provided informed consent prior to participation. The trial was conducted in compliance with the principles of good clinical practice and the Declaration of Helsinki (1996). The RCT was registered prospectively with ISRCTN (Reference ISRCTN16562415). All authors had access to the study data and reviewed and approved the final manuscript.

Participants were naïve to the LFD and did not have any medical condition that might impact gut microbiota (e.g. inflammatory bowel disease, celiac disease, diabetes) or severe psychiatric illness or current eating disorder. Other details of the inclusion and exclusion criteria are provided in the original report.[12] Patients that violated the protocol during the trial were withdrawn and excluded from *per protocol* analysis.

2.2 | Study protocol

The study protocol has been described in detail.[12] Briefly, for 1-week patients recorded their habitual dietary intake and answered the global symptom question ('Over the past 7-day, do you feel that you have had adequate relief of your IBS symptoms?') before attending their baseline appointment where they provided a stool and urine sample and were randomised using computerised randomisation with blocks of six, stratified for IBS-D and sex. Patients were randomised in a 1:1:1 ratio to the three groups by a researcher not otherwise involved in this study.

Patients were instructed to follow either a sham diet (designed to mimic the complexity of a restriction diet but to not alter nutrient, fibre or FODMAP intake, and thus be a placebo for dietary advice)[12] or the low FODMAP diet (single-blinded) and take either a prebiotic or placebo supplement (double-blinded) daily for 4-week. Allocation to diet and supplement was masked as described throughout data collection, laboratory analysis and data input. In the final week of the trial, the same 1-week diet diary was completed as at baseline and the global symptom question data, stool and urine samples were collected.

2.3 | Outcomes

Clinical response ('adequate symptom relief') at 4 weeks was used to classify participants as responders or non-responders. Stool *Bifidobacteria* species (quantified using fluorescent in situ hybridisation (FISH)), faecal microbiota abundance (16S rRNA sequencing), faecal SCFA (gas–liquid chromatography) and VOC (gas chromatography–mass spectrometry, GC-MS) and urine metabolites (^{1}H NMR) were compared between responders and non-responders to each intervention at baseline and 4-week to identify features that could predict or support mechanisms of response to dietary intervention in IBS.

Nutrient, energy and FODMAP intake were calculated using Nutritics® and bespoke software, respectively (Monash University).

2.3.1 | Faecal and urine analysis

A whole fresh stool sample was collected in a sealable sterile bag and placed immediately on ice, patients were asked to deliver the sample to the laboratory within 1 h for processing at the end of the study visit. Samples were homogenised in a stomacher for 4 min before aliquots were stored at −80°C until analysis. Values were adjusted for stool water content by drying a known weight of stool at ~100°C for 24 h or until a constant weight was achieved within 0.01 g.

FISH was performed using a previously described method.[21] Briefly, prior to storage, 50% w/v glycerol was added to the stool aliquot to preserve cells during freeze/thaw. Bacterial cells were extracted from the stool and fixed in paraformaldehyde before hybridisation with fluorescent oligonucleotide DNA probes specific for bifidobacteria (Bif164[22]) and total bacteria (EUB338, EUB338II and EUB338III[23]). Cells were counted manually on a Nikon Eclipse E400 microscope at 1000 magnification by an independent researcher blinded to treatment allocation.

Sequencing was performed by amplification of the V4 region of bacterial 16S rRNA using PCR and Illumina adapter sequences using an Illumina MiSeq platform and a custom pipeline based on Quantitative Insights Into Microbial Ecology (QIIME) 1.9.0, Greengenes was used to assign taxonomy.[24] Both α-diversity (Chao-1) and β-diversity (Bray-Curtis) were measured. A 95000 rarefaction threshold and a filter to remove lower abundance OTUs with a minimum fraction count below 0.0005 were applied before taxonomy assignment.[25] To identify key differences in microbial relative abundance, any genus with <1% abundance in all patient samples was removed before statistical analysis. Differences between responders and non-responders were assessed with non-parametric testing.

Faecal SCFA were extracted using buffer (0.1% mercury, 1% phosphoric acid with 0.0045% 2,2-dimethylbutyric acid internal standard (Sigma)) and quantified using gas-liquid chromatography performed on a 7890A gas chromatograph (Agilent Technologies) and a calibration curve using the Agilent Chromatogram database (Agilent Technologies), using standard techniques.[26]

Volatile organic compound (VOC) analysis was performed using gas-chromatography mass-spectrometry (GC-MS) on a PerkinElmer Clarus 500 GC-MS quadrupole benchtop system (Beaconsfield) and Combi PAL auto-sampler (CTC Analytics) for all stool samples collected at baseline and week-4. Prior to GC–MS analysis VOCs were extracted from the headspace of 450–500 mg of stool in 10 mL headspace vials (Sigma-Aldrich) using solid phase micro-extraction (SPME). A SPME fibre coating of DVB-CAR-PDMS 50/30 µm (1 cm) (Sigma-Aldrich) was used. The optimised SPME extraction method and GC-MS conditions were the same as previously published by Reade et al.[19] Individual VOCs had to be present in at least 50% of samples in at least one experimental condition to remain in the analysis.

Urine metabolites were analysed using hydrogen nuclear magnetic resonance (1H NMR). The first urine sample of the day (midstream) was collected, and 1 mL aliquots were stored at −80°C until analysis. A 2:1 urine: phosphate buffer (0.2 M sodium dibasic, 0.05 M sodium phosphate monobasic dihydrate, 2 mM sodium azide in 50:50 MilliQ H_2O:deuterium oxide containing 1 mM sodium 3-(trimethylsilyl)propionate-d4) was prepared (adapted from a previous study[27]). 1H NMR was performed on a Bruker Avance II 700 NMR spectrometer (Bruker BioSpin) under automation at 298 Kelvin and 700 MHz. Spectra were acquired into Topspin™ software (Bruker Biospin). Spectra were normalised and aligned with MVAPACK.[28]

2.4 | Statistical analysis

Statistical analysis for faecal and urine metabolites was performed *per protocol* (all participants who provided samples) as microbiota and metabolite analysis was dependent on sample provision, clinical response data were carried forward for participants that did not complete the study to give a full set of baseline data for exploring response prediction. Categorical data are presented as n (%) and compared using the χ^2 test, continuous data are presented as mean (SD) or median (IQR), and *t* tests or non-parametric analysis was used for all pairwise comparisons. Differences were considered significant where $p \le 0.05$. Statistical analysis was performed using IBM SPSS Statistics version 24.0, and adjustments for multiple comparisons for the microbiota data were performed using R Core Team (2022).

VOC data were processed using Automated Mass Spectral Deconvolution System (AMDIS-version 2.71, 2012) coupled to the National Institute of Standards and Technology (NIST) mass spectral library (version 2.0, 2011) to putatively identify VOCs. The R package Metab[29] was used to align data. Data were processed using Metaboanalyst[30] and VOC data were log-transformed (natural log), and missing values were replaced with 1. Principal component analysis (PCA) and heatmaps were used to visually compare VOC profiles between groups and differences in VOC abundance between groups were evaluated by *t* test, with *p*-values corrected for multiple comparisons.

VOCs were selected based on patterns in the unsupervised analysis and *t* test results to include in a ROC curve model to classify

responders and non-responders in the LFD group. The sensitivity and specificity values were calculated based on an average of the predicted class probabilities of each sample made across 100 cross-validations. *p*-values for ROC curves were calculated based on 100 permutations as described elsewhere.[31] Finally, a cluster of related VOCs that appeared predictive of response to diet was selected and the sensitivity, specificity, negative and positive predictive values of this cluster were calculated.

Urine metabolite profiles were compared using supervised orthogonal partial-least squared discriminant analysis (OPLS-DA) using bespoke software.[32] S-plots were used to identify the spectral coordinates of metabolites that most contributed to the separation between groups. The coordinates were then assigned to known metabolites using Chenomx® software.

3 | RESULTS

3.1 | Recruitment and clinical response

Sixty nine patients were randomised in the study between July 2015 and July 2016. A total of 130 patients were screened of whom 61 were excluded and 69 randomised to either control, LFD or LFD/GOS groups. At 4 weeks there was a significant difference in the number of clinical responders between groups with 7/23 (30%) in control, 11/22 (50%) in LFD and 16/24 (67%) in the LFD/B-GOS group (χ^2 (2, $N = 69$) = 6.175, $p = 0.048$) (Figure 1). Six patients did not complete the study and were categorised as non-responders for intention to treat analysis. No participants reported withdrawal due to adverse GI effects of diet or supplements.

3.2 | Demographics, nutrition and FODMAP intake in responders and non-responders

Baseline demographics, IBS subtype or medication use were not different between responders and non-responders in any group (Table 1).

At baseline, there were no differences in energy or macronutrient or FODMAP intake between responders and non-responders in the control and LFD groups, although responders had lower fibre (AOAC) and total oligosaccharide intake than non-responders in the LFD/B-GOS group (Table 2).

At the end of trial, the control group responders had lower lactose and GOS intake than non-responders. There were no differences between responders and non-responders in the LFD group, and LFD/B-GOS group responders had lower sorbitol intake than responders (Table 2).

3.3 | Microbiota endpoints

The average time from voiding to processing of stool was 72.4 min, and there were no significant differences in stool processing times between responders and non-responders in any group (Table S1).

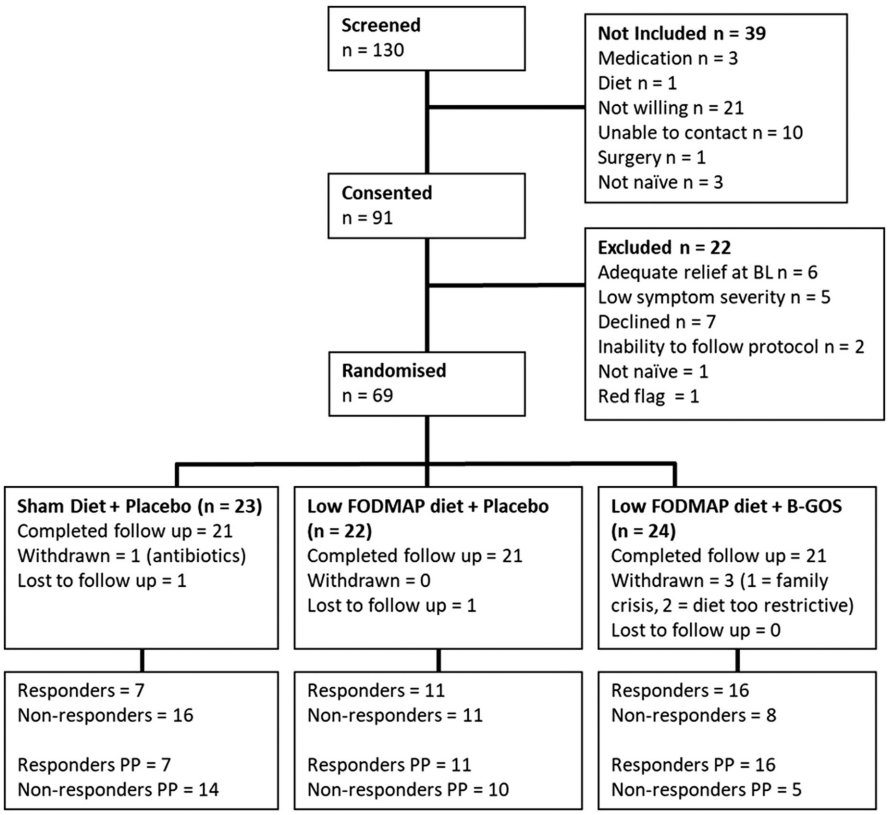

FIGURE 1 CONSORT flow diagram. PP, per protocol.

TABLE 1 Baseline demographics of responders and non-responders to sham dietary advice (control) low FODMAP dietary advice (LFD) or low FODMAP dietary advice plus B-GOS prebiotic (LFD/B-GOS).

	Control			Low FODMAP diet (LFD)			Low FODMAP plus B-GOS (LFD/B-GOS)		
	Responders ($n=7$)	Non-responders ($n=16$)	p-value*	Responders ($n=11$)	Non-responders ($n=11$)	p-value*	Responders ($n=16$)	Non-responders ($n=8$)	p-value*
Female, *n* (%)	3 (43)	9 (56)	0.667	7 (63.6)	6 (54.5)	0.665	9 (56.3)	4 (25.0)	0.772
Age, years, mean (SD)	26.7 (4.8)	31.7 (10.9)	0.262	40 (11)	37 (8)	0.530	32 (8)	35 (8)	0.363
BMI, kg/m^2, mean (SD)	23.6 (1.9)	24.2 (4.7)	0.735	26.5 (3.8)	25.9 (4.9)	0.751	24 (4.5)	23.4 (3.4)	0.764
White British, *n* (%)	4 (57)	10 (63)	1.000	4 (36.4)	6 (54.5)	0.392	11 (68.8)	7 (43.8)	0.317
IBS-D, *n* (%)	5 (71)	10 (63)	1.000	7 (63.6)	8 (72.7)	0.647	10 (62.5)	5 (31.3)	1.000
Hypermobile, *n* (%)	4 (57)	5 (31)	0.363	2 (18.2)	3 (27.3)	0.611	4 (25.0)	0 (0.0)	0.121
Medication, *n* (%)	4 (57)	8 (50)	1.000	6 (54.5)	8 (72.7)	0.375	9 (56.3)	5 (31.3)	0.770
Analgesia	1 (14)	2 (12.5)	1.000	0 (0.0)	2 (18.2)	0.138	2 (12.5)	1 (6.3)	1.000
Anti-diarrhoea	0 (0)	1 (6)	1.000	0 (0.0)	2 (18.2)	0.138	1 (6.3)	2 (12.5)	0.190
Anti-spasmodic	0 (0)	1 (6)	1.000	0 (0.0)	1 (9.1)	0.306	2 (12.5)	1 (6.3)	1.000

*p-values are the result of t test for continuous data and χ^2 test for categorical data.

There was no difference in absolute abundance (log10 cells/g dry weight) of faecal Bifidobacteria between responders and non-responders in any of the groups at baseline or 4 weeks. Change in absolute abundance (log10 cells/g dry weight) of faecal Bifidobacteria between responders and non-responders was only observed in the LFD/B-GOS group, responders had a significantly greater reduction in Bifidobacteria (mean: −0.5%, SD: 0.3) than non-responders (0.0% (0.3)) ($p=0.008$).

Phylum and genus level data (relative abundance, 16S rRNA sequencing) were compared at baseline in the control (responders = 6, non-responders = 10), LFD (responders = 10, non-responders = 8), and LFD/B-GOS (responders = 14, non-responders = 5) groups. There were no differences in any diversity measures between responders and non-responders in any group (data not shown). Differential abundance testing (non-parametric) between responders and non-responders showed differences at baseline, in delta and at end of trial; however, none of the differences described below were significant when corrected for multiple testing.

At baseline control group responders had higher (median: 2.4%, IQR: 1%) levels of one genera in the Lachnospiraceae family than non-responders (1.3%, 0.7%, $p=0.004$, $q=0.816$) and higher Bilophila (0.1%, 0.2%) than non-responders (0.05%, 0.1%, $p=0.044$, $q=0.906$). In the LFD group, responders had higher (0.3%, 0.4%) Streptococcus than non-responders (0.2%, 0.1%, $p=0.048$, $q=0.927$). In the LFD/B-GOS group, responders had lower (0%, 0%) Megamonas than non-responders (2.9%, 18.8%, $p=0.002$, $q=0.408$).

Analysis of change in microbiota showed that the phyla Verrucomicrobia changed less in responders (0%, 0.2%) than non-responders (−0.3%, 1%, $p=0.025$, $q=1$) in the LFD group. There were differences in change in genera between responders and non-responders in each group and these are presented in Figure 2.

At the end of trial, the control group responders had higher Coprococcus (7.5%, 1.9%) than non-responders (5.3%, 3.9%, $p=0.045$, $q=0.906$). In the LFD group, a member of the Clostridiaceae family was lower in responders ($n=11$) (0.0%, 0.0) than non-responders ($n=9$) (0.0%, 0.2, $p=0.027$, $q=927$). In the LFD/B-GOS group, responders had lower (0%, 0%) Megamonas than non-responders (3.9%, 21.1%, $p=0.004$, $q=0.408$); lower (0%, 0%) Serratia than non-responders (0%, 0%, $p=0.046$, $q=0.754$) and lower Acidamonococcus (0%, 0%) than non-responders (0.1%, 0.4%, $p=0.048$, $q=0.754$).

3.4 | Faecal short-chain fatty acids

At baseline, there were no differences in faecal short-chain fatty acids (mg/100 g dry weight) between responders and non-responders in the control group (Table 3).

In the LFD group, propionate was higher in responders (mean: 771.3, SD: 743.6) than non-responders (381.1, 359.7, $p=0.009$) at baseline, and total SCFA were higher in responders (3393.8, 1731.0) than non-responders (2362.7, 2085.6) though this did not reach statistical significance ($p=0.053$). A ROC curve determined that baseline propionate discriminated between responders and non-responders to LFD (AUC: 0.85, 95% CI: 0.64–1.0; $p=0.009$) with a sensitivity of 91%, specificity of 89%, positive predictive value (PPV) 91% and negative predictive value (NPV) of 89%.

In the LFD/B-GOS group responders had higher isobutyrate (73.5, SD 52.6 vs. 26.8 SD: 12.1, $p=0.008$) and isovalerate (104.9, SD:

TABLE 2 Baseline and end of trial (4-week) energy, nutrient and FODMAP intake compared between responders and non-responders to sham dietary advice (control) low FODMAP dietary advice (LFD) or low FODMAP dietary advice plus B-GOS prebiotic (LFD/B-GOS).

	Control			Low FODMAP diet (LFD)			Low FODMAP plus B-GOS (LFD/B-GOS)		
Intakes per day, mean (SD)	Responder ($n=7$)	Non-responder ($n=14$)	p-value[a]	Responder ($n=11$)	Non-responder ($n=10$)	p-value[a]	Responder ($n=16$)	Non-responder ($n=5$)	p-value[a]
Baseline									
Energy (kcal)	1849 (526)	1985 (578)	0.607	2041 (501)	1827 (467)	0.326	1997 (476)	2379 (579)	0.152
Total protein (g)	82.6 (22.4)	90.1 (27.7)	0.545	84.4 (26.0)	75.9 (19.8)	0.412	81.1 (29.0)	95.7 (46.0)	0.403
Fat (g)	77.2 (31.7)	78.3 (24.2)	0.928	82.5 (18.6)	72.9 (18.2)	0.244	83.4 (21.3)	94.3 (22.3)	0.335
Carbohydrate (g)	172.3 (43.7)	202.6 (75.9)	0.343	212.5 (66.9)	188.6 (55.1)	0.385	208.4 (60.3)	252.3 (54.7)	0.164
NSP englyst (g)	12.2 (6.3)	13.0 (4.3)	0.741	15.7 (5.7)	15.0 (6.3)	0.769	13.2 (4.3)	17.5 (6.4)	0.93
Fibre AOAC (g)	16.6 (8.3)	19.2 (6.1)	0.417	21.4 (7.2)	19.2 (7.7)	0.491	18.5 (5.3)	26.1 (10.0)	**0.035**
Total FODMAPs[b]	15.30 (3.75)	17.84 (5.38)	0.271	16.80 (7.21)	17.14 (5.35)	0.903	24.06 (14.24)	20.40 (9.16)	0.599
Fructans (g/d)	0.24 (0.36)	0.37 (0.44)	0.527	0.23 (0.26)	0.20 (0.18)	0.784	0.18 (0.25)	0.48 (0.64)	0.131
Oligos (g/d)	0.62 (0.31)	0.63 (0.37)	0.971	0.68 (0.29)	0.54 (0.31)	0.288	0.48 (0.22)	0.93 (0.39)	**0.004**
Lactose (g/d)	0.66 (0.64)	0.85 (0.54)	0.491	0.67 (0.64)	1.20 (1.30)	0.247	2.87 (3.03)	0.74 (0.71)	0.141
Sorbitol (g/d)	6.73 (4.88)	7.22 (3.71)	0.801	7.14 (4.04)	6.01 (4.90)	0.569	5.44 (4.25)	7.17 (2.98)	0.412
Mannitol (g/d)	0.33 (0.24)	0.36 (0.41)	0.893	0.48 (0.41)	1.03 (1.60)	0.292	0.55 (0.68)	0.77 (0.80)	0.558
GOS (g/d)	2.66 (1.82)	2.58 (1.17)	0.899	2.55 (1.04)	2.03 (1.09)	0.273	2.51 (0.98)	3.31 (1.54)	0.182
Excess fructose (g/d)	4.00 (1.13)	5.84 (2.83)	0.118	5.04 (2.77)	6.15 (2.62)	0.358	12.02 (10.75)	7.02 (4.92)	0.332
End of trial									
Energy (kcal)	1689 (678)	1703 (472)	0.957	1538 (455)	1696 (672)	0.53	1743 (572)	1629 (336)	0.679
Total protein (g)	72.5 (43.3)	81.9 (23.6)	0.522	71.9 (25.3)	73.9 (29.3)	0.872	75.0 (23.5)	81.8 (34.0)	0.618
Fat (g)	72.0 (45.5)	68.9 (17.7)	0.823	66.8 (30.1)	69.1 (35.8)	0.875	73.3 (28.5)	71.4 (21.5)	0.889
Carbohydrate (g)	153.2 (40.2)	159.0 (44.6)	0.777	142.2 (44.6)	162.0 (59.4)	0.395	174.3 (77.3)	133.2 (24.0)	0.263
NSP englyst (g)	12.3 (8.4)	11.9 (3.9)	0.885	10.2 (5.0)	12.5 (5.7)	0.326	11.1 (3.9)	9.9 (4.4)	0.582
Fibre AOAC (g)	16.9 (11.2)	16.9 (6.1)	0.987	16.9 (8.6)	18.1 (8.8)	0.75	17.9 (7.6)	15.8 (8.2)	0.589
Total FODMAPs[b]	12.03 (5.83)	15.13 (6.89)	0.321	6.46 (6.17)	10.84 (7.95)	0.173	10.99 (12.07)	9.23 (4.21)	0.755
Fructans (g/d)	0.07 (0.08)	0.26 (0.26)	0.082	0.05 (0.13)	0.01 (0.03)	0.454	0.03 (0.08)	0.07 (0.16)	0.461
Oligos (g/d)	0.45 (0.27)	0.63 (0.30)	0.193	0.09 (0.09)	0.08 (0.12)	0.824	0.15 (0.22)	0.05 (0.04)	0.339
Lactose (g/d)	0.22 (0.13)	0.87 (0.78)	**0.044**	0.07 (0.12)	0.23 (0.48)	0.286	1.13 (2.30)	0.01 (0.02)	0.298
Sorbitol (g/d)	6.82 (4.41)	5.32 (4.27)	0.461	3.20 (5.07)	4.50 (5.21)	0.57	2.72 (2.89)	7.43 (3.69)	**0.008**
Mannitol (g/d)	0.30 (0.56)	0.35 (0.49)	0.822	0.06 (0.14)	0.07 (0.09)	0.849	0.11 (0.18)	0.03 (0.03)	0.321
GOS (g/d)	1.59 (0.72)	2.42 (0.73)	**0.023**	0.34 (0.26)	0.57 (0.57)	0.236	0.46 (0.35)	0.22 (0.07)	0.148
Excess fructose (g/d)	2.58 (2.14)	5.28 (4.72)	0.17	2.67 (2.12)	5.38 (5.31)	0.134	6.39 (8.37)	1.42 (1.42)	0.21

Abbreviations: AOAC, association of official analytical chemists; NSP, non-starch polysaccharides.

[a]p-values compare responders to non-responders in each group using a t test, p is significant if <0.05.

[b]Total FODMAPs are calculated as the sum of individual carbohydrates including excess fructose (not total fructose).

117.7 vs. 35.2, SD 18.8, $p=0.010$) at baseline than non-responders, respectively. A ROC curve showed that both baseline isobutyrate (AUC: 0.90, 95% CI: 0.77–1.0; $p=0.008$, sensitivity 100%, specificity 63%, PPV 81%, NPV 100%) and isovalerate (AUC: 0.89, 95% CI: 0.74–1.0; $p=0.010$, sensitivity 100%, specificity 82%, PPV 100%, NPV 82%) showed excellent discrimination between responders and non-responders to LFD/B-GOS.

At the end of trial, responders had lower valerate, isobutyrate and isovalerate than non-responders in the control group, there were no differences between responders and non-responders in the LFD group, and both isobutyrate and isovalerate remained higher in responders than non-responders in the LFD/B-GOS group (Table 3).

3.5 | Faecal volatile organic compounds

A PCA analysis of VOC compounds at baseline demonstrated good separation of responders from non-responders in the LFD group but not in the control or LFD/B-GOS groups (Figure 3). A list of VOCs responsible for driving samples in the positive and negative

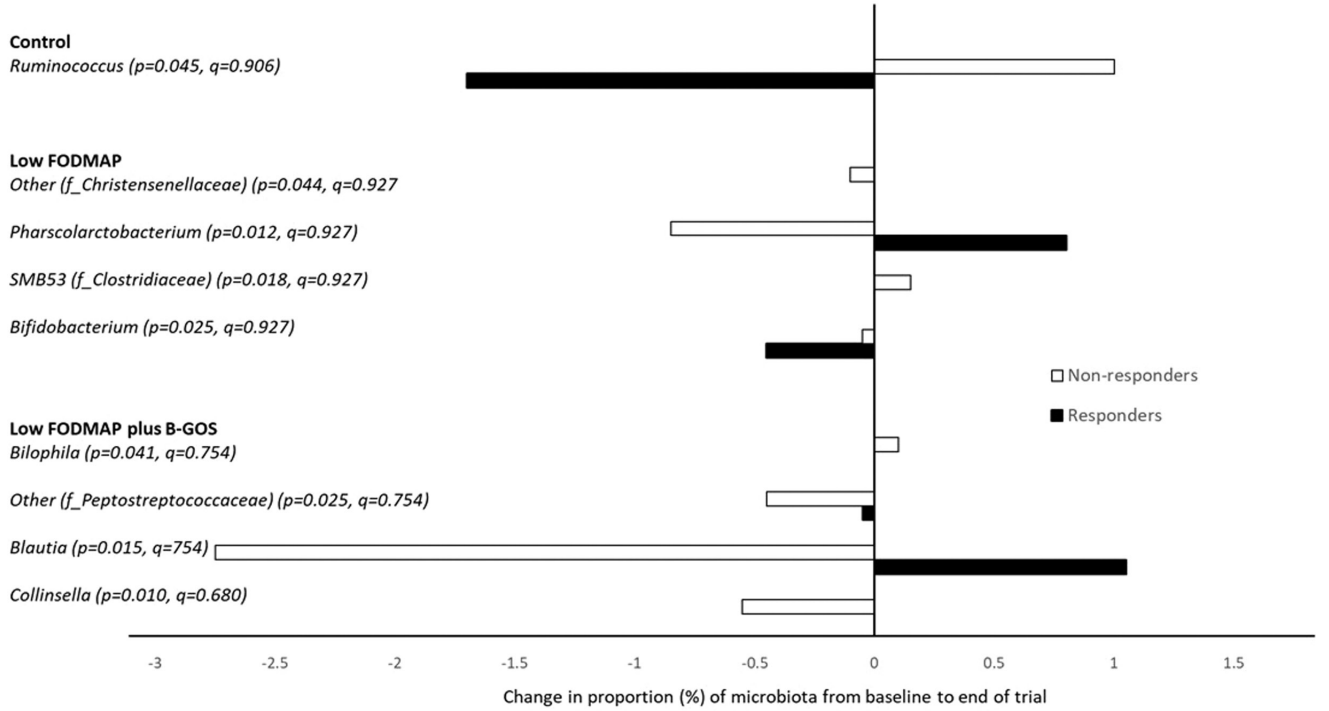

FIGURE 2 Change in microbiota from baseline to end of trial compared between responders and non-responders. Only changes that reached statistical significance prior to adjustment for multiple testing ($p < 0.05$) are included in the figure. Delta data are presented in the figure (median, inter-quartile range) as follows: Control: *Ruminococcus* responders −1.7% (3.4%), non-responders 1% (3.1%). Low FODMAP group: *Other* (f_ Christensenellaceae) responders 0% (0.7%), non-responders −0.1% (0.5%); *Phascolarctobacterium* responders 0.8% (4.5%), non-responders −0.9% (8.4%); *SMB53* (family *Clostriciaceae*) responders 0% (0%), non-responders 0.2% (0.3%); *Bifidobacterium* responders −0.5% (1.4%), non-responders −0.1% (1.1%). Low FODMAP/B-GOS group: *Bilophila* responders 0% (0%), non-responders 0.1% (0.2%); Other (f_*Peptostreptococcaceae)* responders −0.1% (0.3%), non-responders −0.5% (0.7%); *Blautia* responders 1.1% (5.5%), non-responders −2.8% (6.2%); *Collinsella* responders 0% (0.2%), non-responders −0.6% (3.8%).

directions of PC1 for the control, LFD, and LFD/B-GOS groups are shown in Table S2. For the LFD group only PCA loading scores and heatmap visualisation (Figures 3 and 4 and Table S2) revealed a specific cluster of similar VOCs responsible. there were three esters of cyclohexanecarboxylic acid that were in greater concentration in responders than non-responders at baseline. At baseline in the LFD group, cyclohexanecarboxylic acid ethyl ester (unadjusted $p = 0.03$); cyclohexanecarboxylic acid propyl ester (unadjusted $p = 0.08$) and cyclohexanecarboxylic acid butyl ester (unadjusted $p = 0.12$) all showed a trend towards being more abundant in the responders. All baseline *t* test results are shown in Table S3. Differences in baseline VOCs lost their significance once corrected for multiple comparisons (Table S3).

A ROC curve determined that combining baseline values of these three cyclohexanecarboxylic acid esters showed excellent discrimination between responders and non-responders (AUC = 0.84, 95% CI: 0.52, 1 $p = 0.04$, sensitivity 80%, specificity 78%, PPV 80%, NPV 78%). Using presence/ absence data, the results were similar: 80% of patients with one or more of these VOCs were responders, and 75% of those without any of them were non-responders. The presence of one or more of these esters had a positive predictive value of 80%.

At end of trial, VOC profiles did not result in any distinct separation of responders and non-responders in any group (Figure 3, Table S4) and univariate analysis did not reveal any significant changes in VOCs between responders and non-responders in any group (Table S5). Further, clustering for the three cyclohexanecarboxylic acid esters was not seen in the LFD responders as they were largely absent at the end of the trial (Figure 4).

3.6 | Urine

There were no differences in urinary metabolomes between responders and non-responders in either the control group or LFD/B-GOS group at either baseline or at end of trial.

In the LFD group, at baseline, the urine metabolome of responders differed from non-responders ($Q_2 = 0.296$ vs. randomised −0.175) (Figure 5). An S-plot identified that the two metabolites that most contributed to this difference were lower concentrations of creatinine and higher concentrations of trimethylamine N-oxide (TMAO) in responders (Figure S1).

In the LFD group, at the end of trial, the urine metabolome of responders differed from non-responders ($Q_2 = 0.485$ vs. randomised −0.203) (Figure 3), an S-plot identified that the two metabolites that most contributed to this difference were lower concentrations of hippurate and higher citrate in responders (Figure S1).

TABLE 3 Faecal short-chain fatty acid (mg/100 g dry weight) at baseline and week 4 comparing responders and non-responders to sham dietary advice (control) low FODMAP dietary advice (LFD) or low FODMAP dietary advice plus B-GOS prebiotic (LFD/B-GOS).

Mean (SD) SCFA values (mg/100 g)	Control			Low FODMAP diet (LFD)			Low FODMAP plus B-GOS (LFD/B-GOS)		
	Responder ($n=5$)	Non-responder ($n=13$)	p-value*	Responder ($n=11$)	Non-responder ($n=9$)	p-value*	Responder ($n=16$)	Non-responder ($n=5$)	p-value*
Baseline									
Acetate	1341.4 (854.0)	1298.3 (683.0)	0.882	1868.5 (827.8)	1342.6 (1134.4)	0.053	1934.9 (2219.7)	1563.6 (1122.0)	0.804
Propionate	421.6 (242.0)	480.9 (323.0)	0.882	771.3 (743.6)	381.1 (359.7)	**0.009**	790.6 (1174.9)	418.2 (183.2)	0.741
Butyrate	365.5 (236.6)	396.8 (303.4)	0.961	578.7 (315.6)	520.6 (624.4)	0.184	663.8 (811.6)	458.9 (490.5)	0.215
Valerate	37.6 (12.6)	60.6 (37.5)	0.218	65.6 (35.7)	40.4 (21.7)	0.102	121.6 (223.8)	35.7 (23.0)	0.058
Isobutyrate	45.4 (14.8)	46.5 (16.6)	0.657	51.4 (23.1)	31.9 (14.0)	0.063	73.5 (52.6)	26.8 (12.1)	**0.008**
Isovalerate	54.6 (11.4)	56.1 (19.9)	0.805	58.3 (24.5)	46.1 (18.8)	0.239	104.9 (117.7)	35.2 (18.8)	**0.010**
Total SCFA	2266.1 (1302.6)	2339.2 (1250.0)	0.961	3393.8 (1731.0)	2362.7 (2085.6)	0.053	3689.3 (4559.1)	2538.4 (1719.8)	0.869
End of trial	Responder (n=7)	Non-responder (n=13)	p*	Responder (n=11)	Non-responder (n=10)	p*	Responder (n=15)	Non-responder (n=5)	p*
Acetate	1734.9 (963.3)	1593.0 (716.6)	0.823	1306.5 (865.4)	1408.0 (1063.1)	0.888	1364.2 (718.3)	1144.5 (733.7)	0.407
Propionate	484.0 (296.2)	634.1 (420.9)	0.551	418.5 (294.1)	428.2 (334.2)	1.000	444.5 (230.2)	353.3 (331.2)	0.106
Butyrate	715.8 (576.4)	542.6 (356.0)	0.654	399.0 (280.7)	435.1 (319.3)	0.622	419.4 (246.2)	240.0 (145.2)	0.106
Valerate	44.5 (21.8)	80.2 (38.8)	**0.030**	50.8 (34.3)	50.2 (32.1)	1.000	63.6 (44.8)	38.7 (27.4)	0.239
Isobutyrate	33.0 (8.4)	58.6 (22.3)	**0.006**	57.8 (29.8)	38.9 (17.0)	0.105	60.3 (27.9)	32.2 (24.0)	**0.040**
Isovalerate	43.0 (12.3)	65.7 (21.6)	**0.011**	67.1 (33.7)	52.7 (19.7)	0.573	71.2 (31.1)	38.0 (20.0)	**0.032**
Total SCFA	3055.1 (1681.1)	2974.3 (1391.7)	0.881	2299.6 (1498.4)	2413.1 (1664.3)	0.944	2423.3 (1208.8)	1846.6 (1237.2)	0.106

Abbreviations: B-GOS, β-galactooligosaccharide; LFD, low FODMAP diet.

*p is significant if <0.05. Kruskal-Wallis test was used for non-normally distributed data set.

4 | DISCUSSION

While it has been established that the LFD is an effective management strategy for IBS, it is not currently possible to predict response. By identifying a specific profile of those patients most likely to have a clinical response to the low FODMAP diet, this study supports previous findings that faecal and urinary metabolites distinguish responders from non-responders to the LFD.[20,33] This study does not support previous findings that faecal microbiota predict response to the LFD,[15,16,34] however, the sample sizes for comparison were small.

Studies have reported conflicting results for faecal microbiota use in predicting response to LFD, and in the current study, no taxa differed between responders and non-responders to the LFD. However, faecal and urine metabolite differences suggest that the function of the microbiota may be of greater importance in determining response. This supports recent analysis of data from 5 separate LFD intervention studies that reported faecal metabolites were more likely predictive of response than microbiota profiles despite sub-analysis indicating that microbes associated with SCFA production (*Ruminococcaceae* UCG-002, *Ruminococcus 1* and *Anaerostipes*) may be more prevalent in those with a greater response to the diet.[33]

At baseline, faecal propionate was higher and total SCFA showed a trend towards being higher in responders to the LFD. These findings may indicate impaired SCFA absorption in responders at baseline, alternatively, they also support previous research indicating that higher saccharolytic bacteria at baseline may be a driver of response,[35,36] with a previous analysis of data from multiple studies demonstrating high colonic methane and SCFA production was predictive of response to low FODMAP diet.[33] A microbiota rich in carbohydrate-fermenting microbes could feasibly be linked to IBS symptoms associated with gas production, as patients with IBS experience increased symptom intensity with peak colonic gas production.[37] Further, analysis of multiple interventional studies has identified that higher baseline SCFA production is associated with a greater symptom response to the LFD.[33] Higher faecal propionate and SCFA at baseline may be a marker for carbohydrate metabolism in the gut. High production of SCFA, and consequently of gas, prior

to intervention, would be reduced by dietary FODMAP restriction and may, therefore, result in symptom response. Overall, our data confirm evidence that microbiota carbohydrate metabolism capacity is an important feature of responsiveness to a low FODMAP diet. Whilst our data do not present evidence of specific taxa with carbohydrate metabolising capacity predicting response to LFD, as other studies have,[34–36] our sample size was limited in being able to detect such differences.

Three cyclohexanecarboxylic acid esters were shown to be highly sensitive and specific in predicting response to the LFD. Further, these specific VOCs have been shown to be more abundant in the stool of IBS patients than in healthy controls or patients with inflammatory bowel disease.[18] In both previous and current research, the three cyclohexanecarboxylic acid esters were present in most stool samples in IBS patients, but interestingly disappeared from many following the LFD. The origin of the cyclohexanecarboxylic acid esters is unknown however they may arise from the esterification of the acid by the various alcohols in the colon.[38,39] The limited sample size of this study did not allow specific correlations between bacterial taxa and the cyclohexanecarboxylic acid esters to be investigated, but this should be the focus of future research. Potentially, the combined presence of the cyclohexanecarboxylic acid or its ester is key, and this warrants further investigation and external validation.

Whilst many patients experienced symptom response to the LFD/GOS intervention (67%), there was no relationship with the cyclohexanecarboxylic acid esters and the reduction of other esters at 4 weeks was not seen. However, few patients in this group had cyclohexanecarboxylic acid esters at baseline and so the study may have been underpowered to detect differences at baseline in these potential biomarkers.

In addition to faecal metabolites, the urinary metabolome differed between responders and non-responders to the LFD but again not in the control or LFD/B-GOS. The separation at baseline was mostly explained by responders having lower creatinine and higher TMAO. Urinary TMAO is higher in humans that eat a higher animal protein diet as it is converted in the liver from gut-microbe generated TMA made from dietary choline or carnitine,[40,41] creatinine may be an indicator of muscle mass and is higher in men than in women.[27] In the current study, there were no demographic or nutrient intake differences between responders and non-responders that would explain these differences in urinary metabolites. However, TMAO is a by-product of gut microbiota metabolism[27] and could be supportive of the theory that responders' metabolites differ due to different metabolic activities of the microbiome at baseline.

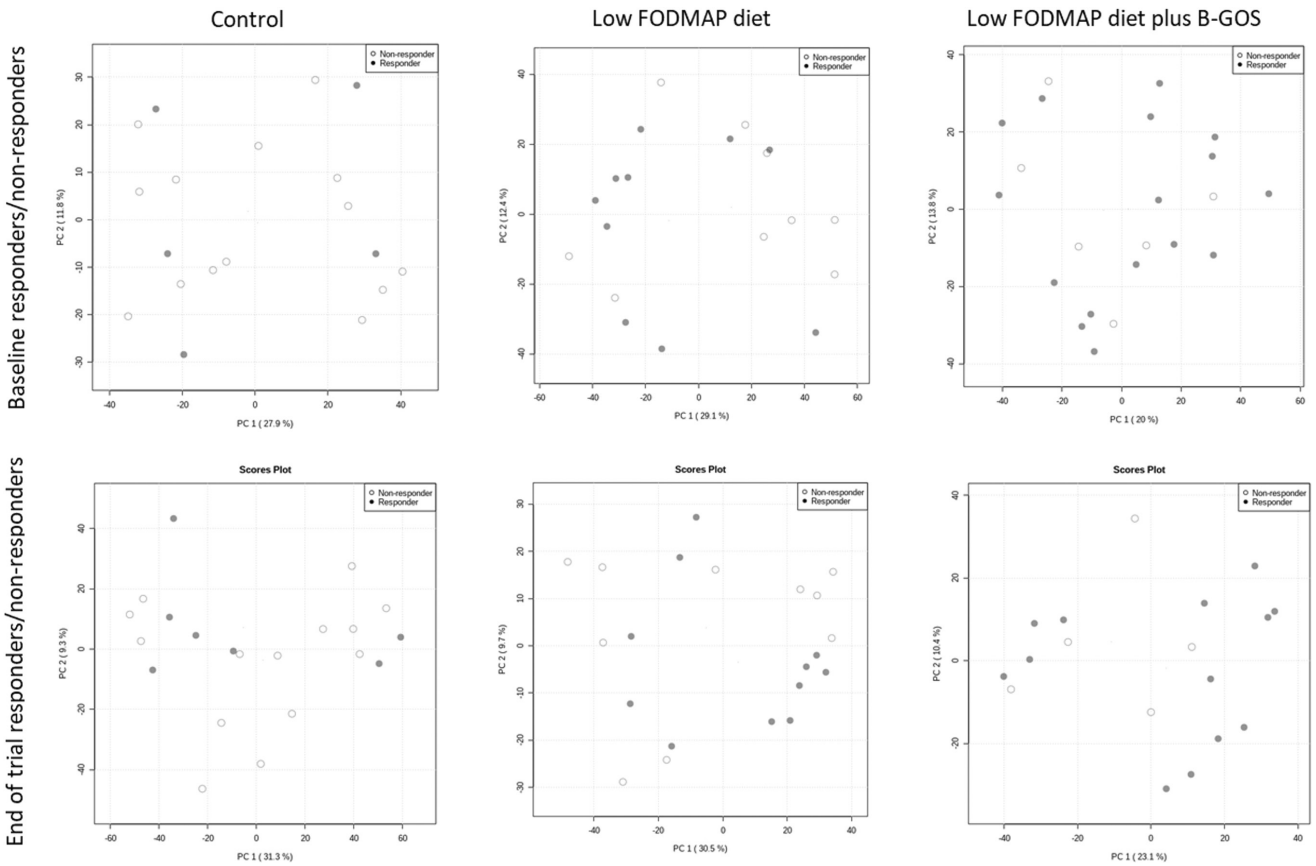

FIGURE 3 Faecal volatile organic compound profiles at baseline and end of trial comparing responders and non-responders to sham dietary advice (control), low FODMAP dietary advice or low FODMAP dietary advice plus B-GOS prebiotic.

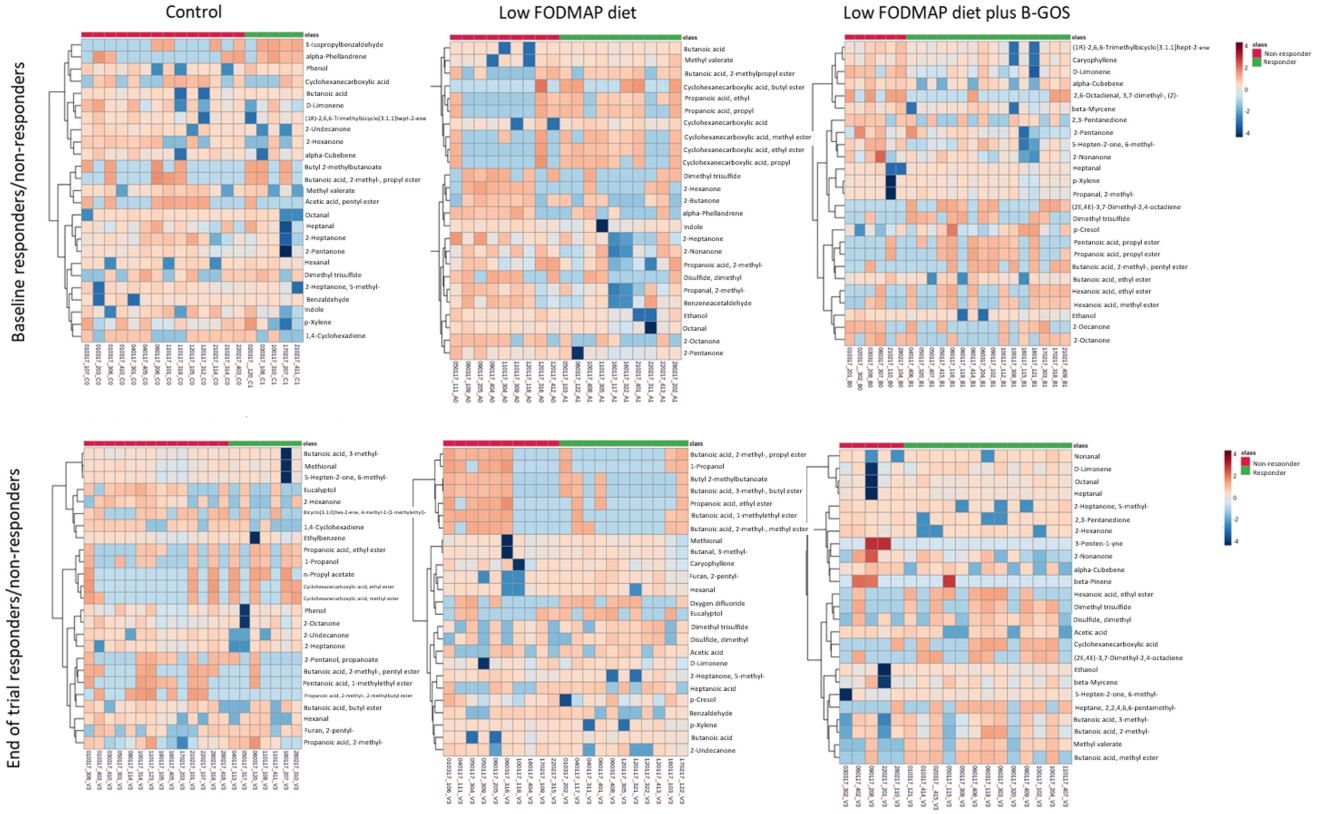

FIGURE 4 Heatmaps showing the 25 faecal volatile organic compounds with the lowest *t* test *p*-values when comparing responders and non-responders to sham dietary advice (control) low FODMAP dietary advice or low FODMAP dietary advice plus B-GOS prebiotic at baseline and the end of trial (4-week). Non-responders are depicted as the 'red' class and responders are depicted as the 'green' class. Blue squares represent a lower value and red squares represent a higher value.

In the LFD group, responders could be distinguished from non-responders by different urine metabolites at baseline and the end of the study. That the distinguishing metabolites differed at baseline and end of the study may provide insight into the mechanism of response. At the end of the study, LFD responders had lower hippurate and higher citrate than non-responders. Hippurate is the glycine conjugate of benzoic acid, is higher in the urine of humans that follow a plant-based diet, and is a marker of consumption of fruit, vegetables and other polyphenolic compounds such as caffeine.[41–43] In the current study, lower hippurate in the responders could indicate lower plant polyphenol intake, however, they also had higher citrate, a biomarker of fruit and vegetable consumption.[43,44] If the difference were due to lower caffeine consumption in responders, this could provide a mechanism for symptom improvement as caffeine is a bowel stimulant.[45] However, the nutritional data does not support this hypothesis either and consequently, the findings remain unexplained.

In general, a number of factors at baseline predicted response to LFD, but not LFD/B-GOS.

In the combined therapy group response to intervention may occur as a result of the LFD (diet alone), the prebiotic (prebiotic alone) or the effect of both. Therefore, the lack of agreement in predicting response between LFD and LFD/B-GOS is that the various factors that may predict response in the latter (diet alone or prebiotics alone or both) obscure the effect of predicting response to LFD alone (diet alone).

4.1 | Strengths and limitations

This study investigated a battery of biological markers to determine those that could predict response to dietary intervention in IBS. The population was well-characterised and monitored carefully. It follows previous work identifying that responders could be separated from non-responders by metabolic profiles,[20,36] and adds that specific metabolites may discriminate response, however, these require external validation. Limitations include the small sample size, which may mean both some legitimate markers were unable to be detected and that there is potential for overfitting of the data. Larger studies should explore a wide range of metabolites including those discovered here. Mathematical modelling between the urine and faecal metabolites, and 16S rRNA sequencing would provide greater insight into how these different features could build a model to predict response to the LFD, however, this would require a larger sample size. Finally, despite patients only being eligible if they were naïve to a low FOAMP diet, average intakes of lactose and some oligosaccharides were relatively low, potentially reflecting self-imposed restriction of some foods prior to participation.

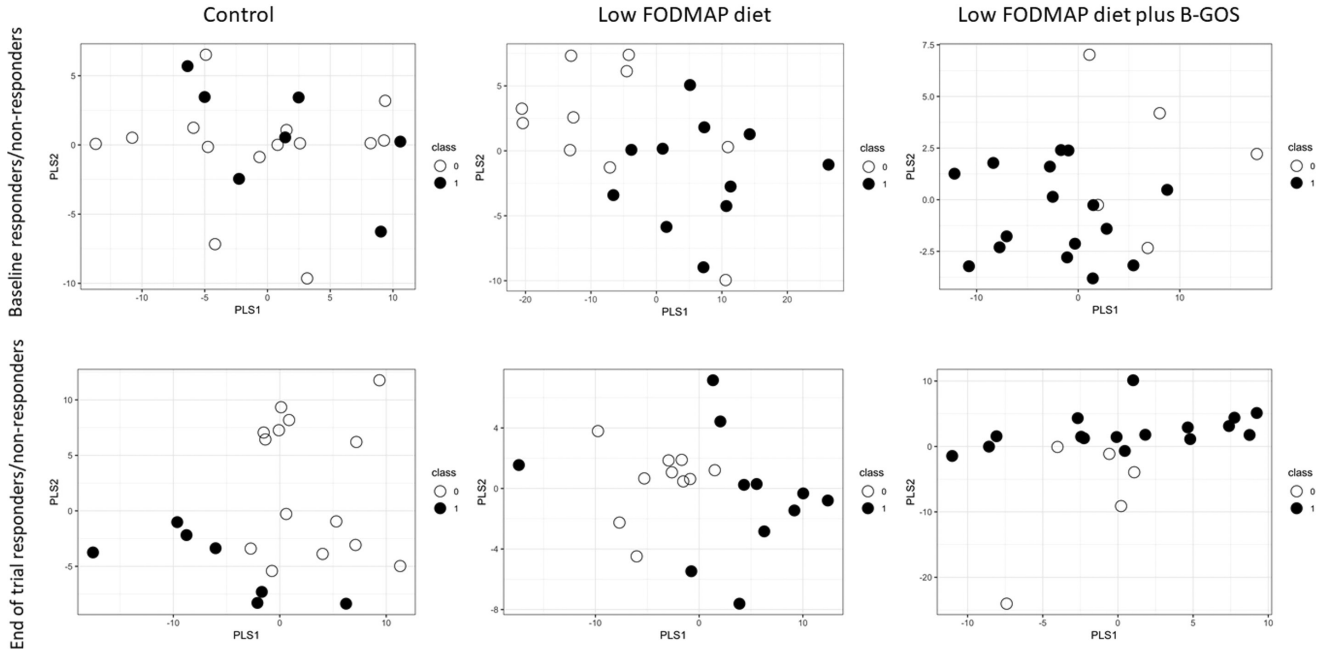

FIGURE 5 Urine metabolite profiles at baseline and week 4 comparing responders and non-responders to sham dietary advice (control) low FODMAP dietary advice or low FODMAP dietary advice plus B-GOS prebiotic.

5 | CONCLUSION

We have demonstrated that markers of microbial activity may predict response to the LFD providing a functional rationale for response. A further adequately powered study is required to validate these predictive markers and may lead to personalised nutrition for patients with IBS.

AUTHOR CONTRIBUTIONS

Bridgette Wilson: Conceptualization (equal); data curation (lead); formal analysis (lead); funding acquisition (equal); investigation (lead); methodology (lead); project administration (lead); writing – original draft (lead). **Tokuwa Kanno:** Formal analysis (equal); writing – review and editing (supporting). **Rachael Slater:** Formal analysis (equal); writing – review and editing (supporting). **Megan Rossi:** Conceptualization (equal); methodology (supporting); writing – review and editing (supporting). **Peter Irving:** Conceptualization (equal); writing – review and editing (supporting). **Miranda C E Lomer:** Conceptualization (equal); writing – review and editing (supporting). **Chris Probert:** Formal analysis (supporting); writing – review and editing (supporting). **A.James Mason:** Formal analysis (supporting); writing – review and editing (supporting). **Kevin Whelan:** Conceptualization (equal); formal analysis (equal); funding acquisition (equal); methodology (equal); supervision (lead); visualization (equal); writing – review and editing (lead). All authors reviewed and approved the final version of the manuscript.

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CONFLICT OF INTEREST STATEMENT

BW was supported by a doctoral research grant from Clasado Biosciences Ltd and is the coinventor of volatile organic compounds in the diagnosis and dietary management of IBS. TK is an employee and shareholder of Johnson and Johnson. MR has received funding from the Almond Board of California, Danone and the International Nut and Dried Fruit Council, and is the coinventor of volatile organic compounds in the diagnosis and dietary management of IBS. PMI was a co-applicant of a research grant from Clasado Biosciences Ltd. MCL was co-applicant of a research grant from Clasado Biosciences Ltd. KW has served as a consultant for Danone, has received research funding from Clasado Biosciences, Almond Board of California, Danone and the International Nut and Dried Fruit Council, and is the coinventor of volatile organic compounds in the diagnosis and dietary management of IBS. CP is the coinventor of volatile organic compounds in the diagnosis and dietary management of IBS. AJM and RS have nothing to disclose.

AUTHORSHIP

Guarantor of the article: Professor Kevin Whelan.

ORCID

Peter M. Irving https://orcid.org/0000-0003-0972-8148
Chris Probert https://orcid.org/0000-0003-4550-0239
Kevin Whelan https://orcid.org/0000-0001-5414-2950

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SUPPORTING INFORMATION

Additional supporting information will be found online in the Supporting Information section.

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