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ORIGINAL ARTICLE

The association of fecal microbiota and fecal, blood serum and urine metabolites in myalgic encephalomyelitis/chronic fatigue syndrome

Christopher W. Armstrong[1] · Neil R. McGregor[2] · Donald P. Lewis[3] · Henry L. Butt[4] ·
Paul R. Gooley[1]

Abstract

Introduction The human gut microbiota has the ability to modulate host metabolism. Metabolic profiling of the microbiota and the host biofluids may determine associations significant of a host–microbe relationship. Myalgic encephalomyelitis/chronic fatigue syndrome (ME/CFS) is a long-term disorder of fatigue that is poorly understood, but has been linked to gut problems and altered microbiota.

Objectives Find changes in fecal microbiota and metabolites in ME/CFS and determine their association with blood serum and urine metabolites.

Methods A workflow was developed that correlates microbial counts with fecal, blood serum and urine metabolites quantitated by high-throughput ^{1}H NMR spectroscopy. The study consists of thirty-four females with ME/CFS (34.9 ± 1.8 SE years old) and twenty-five non-ME/CFS female (33.0 ± 1.6 SE years old).

Results The workflow was validated using the non-ME/CFS cohort where fecal short chain fatty acids (SCFA) were associated with serum and urine metabolites indicative of host metabolism changes enacted by SCFA. In the ME/CFS cohort a decrease in fecal lactate and an increase in fecal butyrate, isovalerate and valerate were observed along with an increase in *Clostridium* spp. and a decrease in *Bacteroides* spp. These differences were consistent with an increase in microbial fermentation of fiber and amino acids to produce SCFA in the gut of ME/CFS patients. Decreased fecal amino acids positively correlated with substrates of gluconeogenesis and purine synthesis in the serum of ME/CFS patients.

Conclusion Increased production of SCFA by microbial fermentation in the gut of ME/CFS patients may be associated with deleterious effects on the host energy metabolism.

Keywords Myalgic encephalomyelitis/chronic fatigue syndrome · Feces · Microbiota · Short chain fatty acids · Energy metabolism · Amino acids

Abbreviations

BCFA	Branched-chain fatty acids
IBD	Irritable bowel disease
ME/CFS	Myalgic encephalomyelitis/chronic fatigue syndrome
NOESY	Nuclear overhauser effect spectroscopy
NMR	Nuclear magnetic resonance
PCA	Principal component analysis
SCFA	Short chain fatty acids
TOCSY	Total correlated spectroscopy

✉ Paul R. Gooley
prg@unimelb.edu.au

[1] Department of Biochemistry and Molecular Biology, Bio21 Molecular Science and Biotechnology Institute, University of Melbourne, 30 Flemington Road, Parkville, VIC 3010, Australia

[2] Faculty of Medicine, Dentistry and Health Sciences, University of Melbourne, Parkville, VIC 3010, Australia

[3] CFS Discovery, Donvale Medical Centre, Donvale, VIC 3111, Australia

[4] Bioscreen (Aust) Pty Ltd, 5 Little Hyde St., Yarraville, VIC 3013, Australia

1 Introduction

In the human gut there are over 100 trillion microbes belonging to over 1000 species and together they form a mass that has the metabolic activity of a "hidden organ" for the host (Guinane and Cotter 2013; Human Microbiome Project 2012). Like any other organ, the gut microbiome develops with the host by a process of modulations with one another; however, the development of the microbiome is greatly influenced by a number of external and environmental factors (Nicholson et al. 2012). These factors create a large diversity of gut microbiome compositions between healthy adult hosts with a limited number of measurable genetic factors that enable the prediction of these compositions (Dorrestein et al. 2014).

Recent research has been conducted on the impact of diet and the gut microbiota on metabolite production and its relevance to disease (Guarner and Malagelada 2003). Understanding the symbiotic relationships of host–microbe and microbe–microbe interactions is complicated as these relationships are altered by reactions to each other making it difficult to deduce an origin of cause and the subsequent effect. In this respect, the detailed systems biology understanding of the impact of individual microbes upon the gut and the host and how they are cultivated remains to be elucidated. To determine the impact of microbes on health, metabolomics offers a practical approach to investigate the host–microbe relationships through the correlations of identified and quantitated bacteria with host metabolites (Li et al. 2008; Schaubeck et al. 2016). The impact of microbes on host health is already being realized through fecal transplant treatments which effectively transplant the diseased "hidden organ" with a healthy donor microbiome. This treatment has been claimed to be successful for treating a number of illnesses including ulcerative colitis (Moayyedi et al. 2015), Metabolic Syndrome (Vrieze et al. 2012), Multiple Sclerosis (Borody et al. 2011) and myalgic encephalomyelitis/chronic fatigue syndrome (ME/CFS) (Borody et al. 2014; Borody and Khoruts 2012).

ME/CFS is a long-term disorder of fatigue that is exacerbated by exercise and not sufficiently relieved by rest. The fatigue is accompanied by a host of other symptoms, including post-exertional malaise and various symptoms of cognitive dysfunction, chronic immune activation, neuroendocrine dysregulation, heightened sensitivity, cardiovascular issues and gut dysbiosis. The disorder is poorly understood, the mechanism of its pathogenesis is likely to be multi-faceted including predisposing, triggering, and maintenance factors (White 2004). The history of research into ME/CFS is littered with findings from studies that have subsequently not been replicated. However, recent metabolomics studies on blood in combination with past metabolite studies have produced a set of consistent factors including: increased oxidative stress, depleted amino acids and lipids, depleted purine metabolites, reduced folate cycle, increased sugars and depletion of TCA cycle metabolites (Armstrong et al. 2015, 2012; Georgiades et al. 2003; Jacobson et al. 1993; McGregor et al. 1996; Naviaux et al. 2016; Niblett et al. 2007; Pall 2002; Suarez et al. 2010; Yamano et al. 2016). Along with increased oxidative stress it has been observed that there are immune anomalies consistent with altered immune activation (Hornig et al. 2015). The chronic aspect of ME/CFS suggests that the pathogenesis may involve an altered state of homeostasis following a trigger event, an autoimmunity issue, a continual pathogen infection or a continual external stimulus. The gut may be involved in these modes of pathogenesis as a few studies have found alterations in the microbe content of fecal samples from ME/CFS patients (Giloteaux et al. 2016; Sheedy et al. 2009).

In this study we present a workflow (Fig. 1) for studying the impact of gut bacteria and metabolites on the host metabolism. We applied this workflow to ME/CFS to elucidate the involvement of the gut in the disorder. By correlating bacterial counts with fecal, blood serum and urine metabolites quantitated by high-throughput ^{1}H NMR spectroscopy we were able to visualize relationships that were relatively consistent across the ME/CFS cohort as compared to non-ME/CFS controls. We found this to be effective in viewing associations between the gut and metabolism of the host and believe that this research has great potential for formulating individual and specific treatment and monitoring for people with gut issues associated to ME/CFS and other illnesses.

2 Materials and methods

2.1 Sample collection

Thirty-four females with ME/CFS (34.9 ± 1.8 SE years old) and twenty-five non-ME/CFS female participants (33.0 ± 1.6 SE years old) were recruited. The ME/CFS group comprised patients that are currently symptomatic and diagnosed as having ME/CFS according to the Canadian guidelines (Carruthers et al. 2003) and were all cared for by the same clinician. None of the subjects were related to one another nor were they ever living together and no difference in BMI was observed between ME/CFS (24.0 ± 0.81 SE) and non-ME/CFS (23.0 ± 0.74 SE) groups. This study was approved by the University of Melbourne human research ethics committee (HREC# 0723086).

All subjects were asked to list their current medications and oral supplements. Prior to fecal sample collection, patients were instructed to cease antibiotic and/or probiotic treatment for 4 and 2 weeks, respectively. Patients collected

a sample of their first morning bowel motion in a fecal container (anaerobic pouch system) with a perforated lid to aid anaerobiosis [achieved by activating Anaero Gen Compact (Oxoid, Thermo Fisher Scientific, Australia)]. Samples were immediately transported to the laboratory in cold conditions (<12 °C) for analysis within 48 h after collection. Laboratory protocol rejects samples subjected to inaccurate collection, transportation, anaerobiosis or refrigeration procedures. Internal quality assurance investigations validated the anaerobic transport and culture methods. After removal from the anaerobic pouch system, all fecal samples were processed within 10–15 min.

2.2 Sample preparation for NMR analysis

A small portion of each fecal sample was homogenized by vortex in deuterated saline at 1:10 w/v before being syringe filtered (0.2 μm, 25 mm diameter) and dispensed into five 1 mL aliquots for storage at −80 °C. All fecal solution samples were prepared for NMR analysis using a liquid–liquid extraction technique (Sheedy et al. 2010). All 59 fecal solution samples were prepared simultaneously. A 250 μL sample of each fecal solution was added to 250 μL ice-cold deuterated chloroform and 250 μL ice-cold deuterated methanol and mixed by vortexing before being left to sit on ice for 15 min. Samples were then centrifuged (16,100×g) at 4 °C for 10 min to produce a biphasic mixture with a hydrophilic phase of water/deuterated methanol and lipophilic phase of deuterated chloroform. A 297 μL sample of the top hydrophilic layer was added to an equal volume of 200 mM sodium phosphate in ^2H_2O (pH 7) and 66 μL of ^2H_2O containing 5 mM DSS and 0.2% (w/v) sodium azide to make a total volume of 660 μL. The sample was then mixed by vortex, centrifuged for 1 min at 13,000 rpm and 550 μL of supernatant was transferred to a 7-inch 5-mm 507-grade NMR tube for NMR analysis. While the apolar layer was discarded, future studies are intended to investigate the lipid profiles from serum and fecal samples.

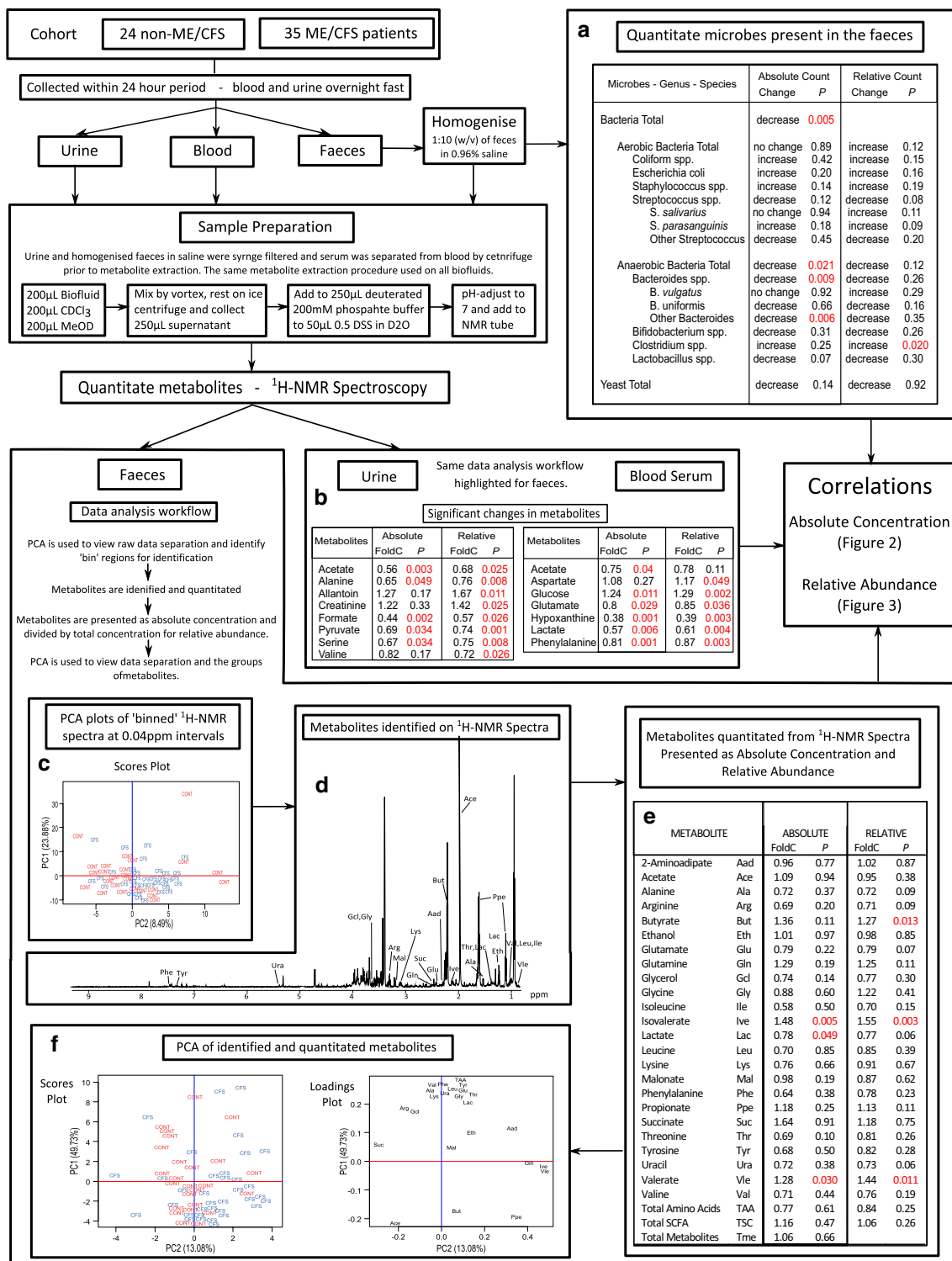## 2.3 Microbial identification and quantification

Between 0.5 and 1.0 g of fecal matter was transferred to 10 mL of 1% glucose-saline buffer and dilutions from 10^{-1} to 10^{-7} of homogenised fecal samples were prepared (Willis 1991). Dilutions (10 and/or 1 μL amounts) were transferred onto Columbia horse blood agar (Oxoid), chromogenic medium (Oxoid), colistin and nalidixic acid blood selective agar (Oxoid), and chloramphenicol–gentamicin selective Sabouraud agar for aerobic incubation. Anaerobic incubation (4 days duration) in anaerobic jars (Oxoid) utilised pre-reduced Columbia horse blood haemin agar and Raka Ray medium. Aerobic media were incubated at 35 °C for 48 h. A stereomicroscope was used to examine both aerobic and anaerobic culture plates for a minimum of 20 min/plate before bacterial identification. Each colony from each medium was microscopically examined and the colony/viable count were quantified for each plate. To assess microbial purity prior to identification, similar morphotypes were sub-cultured onto horse blood agar.

Following overnight purity checks, index bacterial colonies were transferred to a target polished steel plate (MSP 96, Bruker Daltonics Inc.) for drying at room temperature. Air-dried samples were subjected to protein extraction with 1 μL 70% formic acid (Sigma). After repeat air-drying under exhaust ventilation, samples were overlaid with 1 μL of matrix solution (saturated solution of α-cyano-4-hydroxycinnamic acid (HCCA) in a mixture of 47.5% 18 mOhm water, 2.5% trifluoroacetic acid, and 50% acetonitrile). Dried samples were analysed using Microflex MALDI-TOF mass spectrometer (Bruker Daltonik GmbH, Leipzig, Germany) equipped with a 60 Hz nitrogen laser. Spectra were recorded in the positive linear mode for the mass range of 2–20 kDa at maximum laser frequency. The MALDI Biotyper 3.0 software package (default settings; Daltonik GmbH, Bremen, Germany) was used to automatically analyse and measure raw spectra without user intervention.

2.4 NMR measurements

All NMR spectra of fecal solutions were collected at 25 °C on an 800 MHz Bruker Avance II US² spectrometer equipped with a 5 mm triple resonance cryoprobe. The 90° pulse width was calibrated and the receiver gain optimized. Standard 1D ^1H spectra were acquired using a 1D NOESY with a relaxation delay of 1.2 s and a mixing time (t_m) of 0.1 s. Water suppression was achieved by presaturation of the water signal during relaxation delay and mixing time. Each spectrum was collected over 32 K of data points and 128 scans with a spectral width of 10.014 ppm. Before Fourier transformation, the Free Induction Decay was multiplied by a 0.3 Hz exponential line broadening and zero-filled. Chenomx v6.1 NMR Suite Professional software package was used to manually phase and baseline correct spectra. The compound libraries in the Chenomx software were used to identify and quantitate metabolites in the ^1H spectra based on their characteristic chemical shifts using 0.5 mM DSS as an internal chemical shift reference at 0 ppm. Twenty-four metabolite identities were quantitated per fecal solution sample. Standard 2D ^1H TOCSY spectra were conducted to confirm the identities of metabolites observed.

Cohort | 24 non-ME/CFS | 35 ME/CFS patients

Collected within 24 hour period - blood and urine overnight fast

Urine | Blood | Faeces

Homogenise
1:10 (w/v) of feces in 0.96% saline

a Quantitate microbes present in the faeces

Microbes - Genus - Species	Absolute Count		Relative Count	
	Change	P	Change	P
Bacteria Total	decrease	0.005		
Aerobic Bacteria Total	no change	0.89	increase	0.12
Coliform spp.	increase	0.42	increase	0.15
Escherichia coli	increase	0.20	increase	0.16
Staphylococcus spp.	increase	0.14	increase	0.19
Streptococcus spp.	decrease	0.12	decrease	0.08
S. salivarius	no change	0.94	increase	0.11
S. parasanguinis	increase	0.18	increase	0.09
Other Streptococcus	decrease	0.45	decrease	0.20
Anaerobic Bacteria Total	decrease	0.021	decrease	0.12
Bacteroides spp.	decrease	0.009	decrease	0.26
B. vulgatus	no change	0.92	increase	0.29
B. uniformis	decrease	0.66	decrease	0.16
Other Bacteroides	decrease	0.006	decrease	0.35
Bifidobacterium spp.	decrease	0.31	decrease	0.26
Clostridium spp.	increase	0.25	increase	0.020
Lactobacillus spp.	decrease	0.07	decrease	0.30
Yeast Total	decrease	0.14	decrease	0.92

Sample Preparation

Urine and homogenised faeces in saline were syrnge filtered and serum was separated from blood by cetnrifuge prior to metabolite extraction. The same metabolite extraction procedure used on all biofluids.

200µL Biofluid, 200µL $CDCl_3$, 200µL MeOD | Mix by vortex, rest on ice centrifuge and collect 250µL supernatant | Add to 250µL deuterated 200mM phospahte buffer to 50µL 0.5 DSS in D2O | pH-adjust to 7 and add to NMR tube

Quantitate metabolites - ^{1}H-NMR Spectroscopy

Faeces

Data analysis workflow

PCA is used to view raw data separation and identify 'bin' regions for identification

Metabolites are idenified and quantitated

Metabolites are presented as absolute concentration and divided by total concentration for relative abundance.

PCA is used to view data separation and the groups of metabolites.

b Urine — Same data analysis workflow highlighted for faeces. — Blood Serum

Significant changes in metabolites

Metabolites	Absolute		Relative	
	FoldC	P	FoldC	P
Acetate	0.56	0.003	0.68	0.025
Alanine	0.65	0.049	0.76	0.008
Allantoin	1.27	0.17	1.67	0.011
Creatinine	1.22	0.33	1.42	0.025
Formate	0.44	0.002	0.57	0.026
Pyruvate	0.69	0.034	0.74	0.001
Serine	0.67	0.034	0.75	0.008
Valine	0.82	0.17	0.72	0.026

Metabolites	Absolute		Relative	
	FoldC	P	FoldC	P
Acetate	0.75	0.04	0.78	0.11
Aspartate	1.08	0.27	1.17	0.049
Glucose	1.24	0.011	1.29	0.002
Glutamate	0.8	0.029	0.85	0.036
Hypoxanthine	0.38	0.001	0.39	0.003
Lactate	0.57	0.006	0.61	0.004
Phenylalanine	0.81	0.001	0.87	0.003

Correlations

Absolute Concentration (Figure 2)

Relative Abundance (Figure 3)

PCA plots of 'binned' ^{1}H-NMR spectra at 0.04ppm intervals

c Scores Plot

Metabolites identified on ^{1}H-NMR Spectra

d

Metabolites quantitated from ^{1}H-NMR Spectra Presented as Absolute Concentration and Relative Abundance

e

METABOLITE		ABSOLUTE		RELATIVE	
		FoldC	P	FoldC	P
2-Aminoadipate	Aad	0.96	0.77	1.02	0.87
Acetate	Ace	1.09	0.94	0.95	0.38
Alanine	Ala	0.72	0.37	0.72	0.09
Arginine	Arg	0.69	0.20	0.71	0.09
Butyrate	But	1.36	0.11	1.27	0.013
Ethanol	Eth	1.01	0.97	0.98	0.85
Glutamate	Glu	0.79	0.22	0.79	0.07
Glutamine	Gln	1.29	0.19	1.25	0.11
Glycerol	Gcl	0.74	0.14	0.77	0.30
Glycine	Gly	0.88	0.60	1.22	0.41
Isoleucine	Ile	0.58	0.50	0.70	0.15
Isovalerate	Ive	1.48	0.005	1.55	0.003
Lactate	Lac	0.78	0.049	0.77	0.06
Leucine	Leu	0.70	0.85	0.85	0.39
Lysine	Lys	0.76	0.66	0.91	0.67
Malonate	Mal	0.98	0.19	0.87	0.62
Phenylalanine	Phe	0.64	0.38	0.78	0.23
Propionate	Ppe	1.18	0.25	1.13	0.11
Succinate	Suc	1.64	0.91	1.18	0.75
Threonine	Thr	0.69	0.10	0.81	0.26
Tyrosine	Tyr	0.68	0.50	0.82	0.28
Uracil	Ura	0.72	0.38	0.73	0.06
Valerate	Vle	1.28	0.030	1.44	0.011
Valine	Val	0.71	0.44	0.76	0.19
Total Amino Acids	TAA	0.77	0.61	0.84	0.25
Total SCFA	TSC	1.16	0.47	1.06	0.26
Total Metabolites	Tme	1.06	0.66		

f PCA of identified and quantitated metabolites

Scores Plot

Loadings Plot

2.5 Data analysis

The workflow used for analysis is displayed in Fig. 1 and is discussed in Sect. 3.1. Prior to statistical analysis all data were assessed for normality. Those metabolites or microbial counts not found to be normally distributed were either log converted in the raw data set or arcsine converted for the relatively distributed data set. T tests and Benjamini–Hochberg adjustments were conducted on the identified fecal metabolites and microbial counts to establish significant metabolites and microbes. Correlation graphs were developed in-house using Microsoft Excel 2010 to overlay

◄Fig. 1 Workflow diagram to analyze fecal microbes and metabolites across blood serum, urine and fecal samples. **a** The change and *P* value (*P*) of live microbe count identified in the fecal samples of ME/CFS patients compared to non-ME/CFS subjects. **b** The significant metabolites in blood serum and urine as fold change (FoldC) and *P* value (*P*) as previously published (Armstrong et al. 2015). **c** The.PCA *plot* shows the separation of the ME/CFS and non-ME/CFS using the area under the *curve* from 250 'bins' (0.04 ppm wide) of the feces NMR spectra. **d** A 1D ^{1}H NMR spectrum of feces and its metabolites, identified metabolites are annotated. **e** The fold change (FoldC) and *P* value (*P*) of metabolites identified within the feces of ME/CFS patients compared to non-ME/CFS subjects. Metabolites were quantitated using Chenomx NMR suite 6.1. Data is presented in both absolute and relative distribution forms. *P* values have been adjusted using the Benjamini-Hochberg equation and significant differences are highlighted in red. **f** PCA *plots* using the relatively distributed blood serum metabolite data present a *scores plot* with separation of the ME/CFS and control cohorts and a loadings plot with the metabolites distribution that contributed to the *scores plot* separation

the correlations within both the ME/CFS and non-ME/CFS to highlight discrepancies.

3 Results and discussion

3.1 The experimental workflow

Depicted in Fig. 1 is the workflow used to observe the relationship between gut microbes, fecal metabolites and metabolites from blood serum and urine. The fecal sample is used to conduct a microbial count (Fig. 1a) and a metabolic analysis along with the serum and urine samples. The serum and urine samples that were used for a metabolic analysis using ^{1}H NMR spectroscopy are discussed in a previous paper (Armstrong et al. 2015).

Metabolite data analysis followed the same workflow for the three separate biofluids but is only depicted in Fig. 1 for feces. A table of the significant metabolites from serum and urine (Fig. 1b) are provided (Armstrong et al. 2015). Unsupervised PCA analysis was conducted on the fecal ^{1}H NMR spectra by using spectral 'binned' data (Fig. 1c) to observe a separation of the ME/CFS and non-ME/CFS cohorts and to highlight all peaks that contributed to that separation for metabolite identification and quantitation. The identification and quantitation of fecal metabolites from peaks on the ^{1}H NMR spectra (Fig. 1d) were conducted using Chenomx software. The statistical significance of fecal metabolites between the ME/CFS and non-ME/CFS groups were analyzed using Student *t* tests and were presented along with fold change between the groups (Fig. 1e). Student *t* tests were also used to analyse the statistical significance of microbes (Fig. 1a) and the serum and urine metabolites (Fig. 1b) between the ME/CFS and non-ME/CFS groups.

Microbes were categorized mostly by genus with *Bacteroides* spp. and *Streptococcus* spp. further identified into several species (Fig. 1a). Bacteria were totaled and subtotaled as aerobic and anaerobic bacteria. The metabolite concentrations and microbial counts are presented in both absolute (or raw) values and relative distribution (% of total) values where the latter accounts for fecal, urine and blood serum dilution factors that vary from sample to sample. Normalising all datasets to the same two data types enables Pearson correlation analyses across all the metabolites and microbes in both absolute (Fig. 2) and relative (Fig. 3) forms. Correlation coefficients (*r*) from Pearson correlation analysis quantitates the strength of two separate factors fluctuating with one another from sample to sample across a whole cohort. The relationship of microbes and fecal metabolites were studied with one another and then with serum and urine metabolites (Figs. 2, 3).

The correlational analyses in Figs. 2 and 3 allow us to observe the association of fecal microbes and metabolites of serum, urine and feces in the two distinct cohorts of ME/CFS and non-ME/CFS. Expectedly, most correlations occurred within the same biofluid; however, there were also a large number of relationships existing in inter-biofluid correlations. Figure 2 shows large discrepancies between the ME/CFS and non-ME/CFS with respect to fecal amino acids and fecal organic acids. These differences are further defined when these fecal metabolites are correlated to serum and urine metabolites. This disturbance in correlations reflects the disparate biochemical signatures that exist between ME/CFS patients and non-ME/CFS control groups as we observed 14 metabolites altered out of a total of 83 identified metabolites (17%) across feces, urine and serum (Fig. 1b, e). These correlations offer information on how these metabolite differences may be related to one another and other metabolites across the host system. Information from these relationships depicted for ME/CFS will be further extrapolated using previous research on metabolism and ME/CFS in the following sections.

3.2 Workflow evaluation through non-ME/CFS control correlations
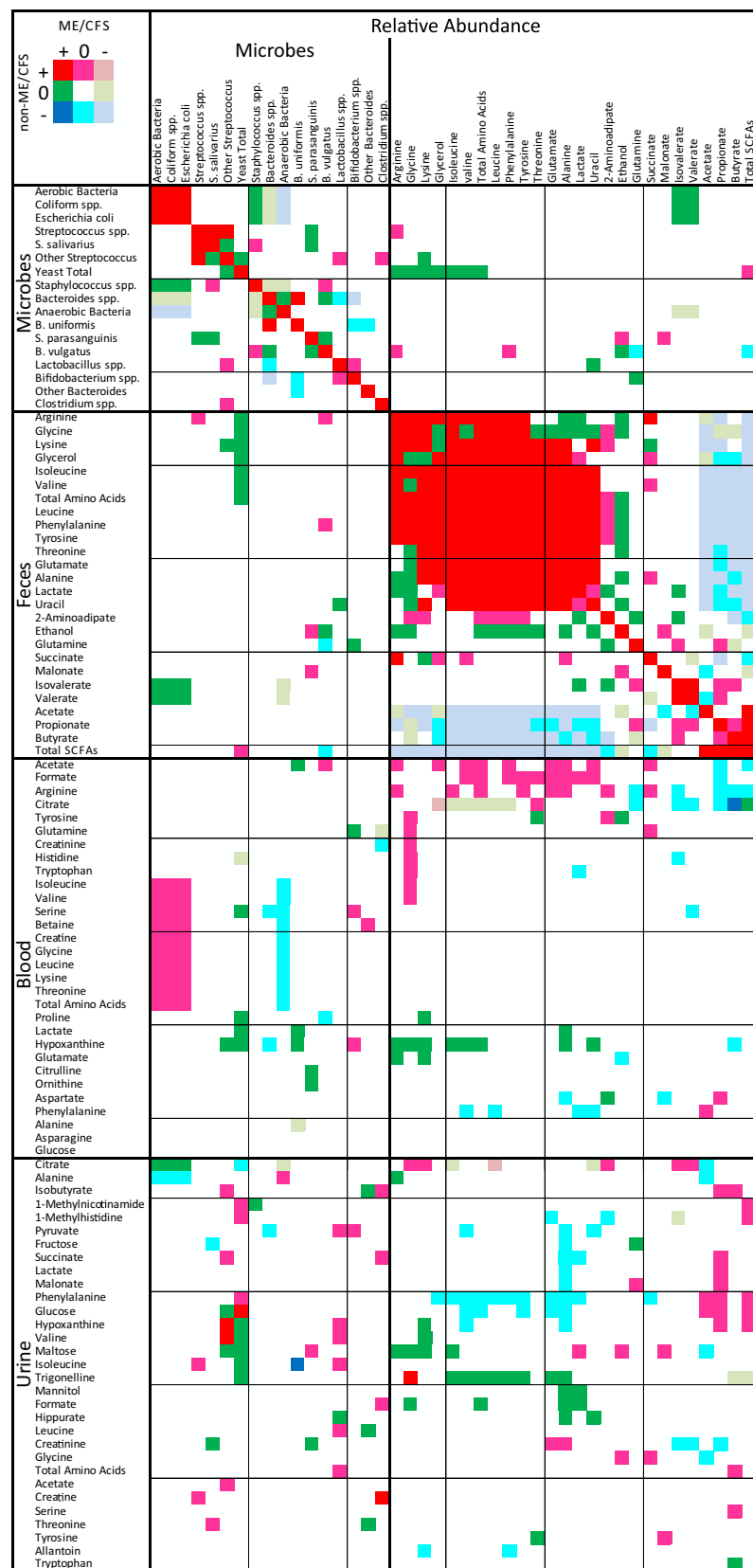
The presented workflow was designed to observe how the gut and the host affect one another by an indirect method using correlations of blood serum and urine metabolites with fecal metabolites and bacteria. The validity of the workflow relies upon correlations in the non-ME/CFS group mirroring previous research.

If we highlight the non-ME/CFS group on its own we see that fecal metabolites positively correlate with one another in the absolute data (Fig. 2), indicating a fluctuation of fecal metabolites depending on the water content and biological material in the feces. Most fecal metabolites, particularly the short-chain fatty acids (SCFA), were negatively correlated with total serum metabolites (Fig. 2).

Fig. 2 Absolute concentration fecal metabolites and microbes correlated with all metabolites in ME/CFS and non-ME/CFS cohorts. The correlations are displayed as *colors* in terms of positive ($r > 0.4$), no correlation ($-0.4 < r < 0.4$), and negative ($-0.4 > r$) correlations in both ME/CFS and non-ME/CFS groups. Dark-colors (*red, green, blue*) are a positive correlation in ME/CFS; light-colors (*light red, light green, light blue*) are a negative correlation in ME/CFS; and fluorescent-colors (fluorescent *pink*, fluorescent *blue*) are no correlation in ME/CFS. *Red* hues (*red*, fluorescent *pink, light red*) are a positive correlation in non-ME/CFS; *blue* hues (*blue*, fluorescent *blue, light blue*) are a negative correlation in non-ME/CFS; and *green* hues (*green, light green*) are no correlation in non-ME/CFS

Fig. 3 Relative abundance fecal metabolites and microbes correlated with all metabolites in ME/CFS and non-ME/CFS cohorts. The correlations are displayed as *colors* in terms of positive (*r* > 0.4), no correlation (−0.4 < *r* < 0.4), and negative (−0.4 > *r*) correlations in both ME/CFS and non-ME/CFS groups. Dark-colors (*red, green, blue*) are a positive correlation in ME/CFS; light-colors (*light red, light green, light blue*) are a negative correlation in ME/CFS; and fluorescent-colors (fluorescent *pink*, fluorescent *blue*) are no correlation in ME/CFS. *Red* hues (*red*, fluorescent *pink, light red*) are a positive correlation in non-ME/CFS; *blue* hues (*blue*, fluorescent *blue, light blue*) are a negative correlation in non-ME/CFS; and *green* hues (*green, light green*) are no correlation in non-ME/CFS

The effects of SCFA according to the literature appear to be consistent here in these correlations of the non-ME/CFS control group (den Besten et al. 2013a, b). Microbial SCFA are an important fuel source for cells of the gut wall and also influence the energy metabolism of the host via their increased incorporation into lipids in adipocytes and their increased oxidation to produce glucose and glycogen in muscle cells and hepatocytes (Fig. 4) (Canfora et al. 2015; den Besten et al. 2013b). After removing the effect of concentration of metabolites from the biofluids by observing the relative abundance (Fig. 3) we still see that the SCFA, except for acetate, are negatively correlated to citrate. Propionate enters the hepatocytes to enhance glucose production via gluconeogenesis (Fig. 4) and was observed here to positively correlate with serum aspartate and urine glucose. These observations suggest, as expected, that fecal SCFA entering the blood may be altering energy metabolism by increasing gluconeogenesis and decreasing lipogenesis.

The bacteria *B. vulgatus* and *S. parasanguinis* positively correlated with most fecal metabolites in the non-ME/CFS indicating that their concentrations are likely to be altered by the volume of water and biological matter (Fig. 2). *S. parasanguinis* is prevalent within the oral cavity and may be indicative of food consumption (Corby et al. 2005), while *B. vulgatus* is one of the most prevalent bacteria found in feces (Rigottier-Gois et al. 2003) and therefore may fluctuate with the content of fecal material. *Clostridium* spp. and *Bacteroides* spp. are major fermenters that produce SCFA (Van den Abbeele et al. 2013), both as expected were observed to positively correlate with SCFA (Fig. 2).

3.3 Significantly altered fecal microbes in patients with ME/CFS

Student *t* tests on the absolute count of microbes observed a significant decrease in total bacteria in ME/CFS patients
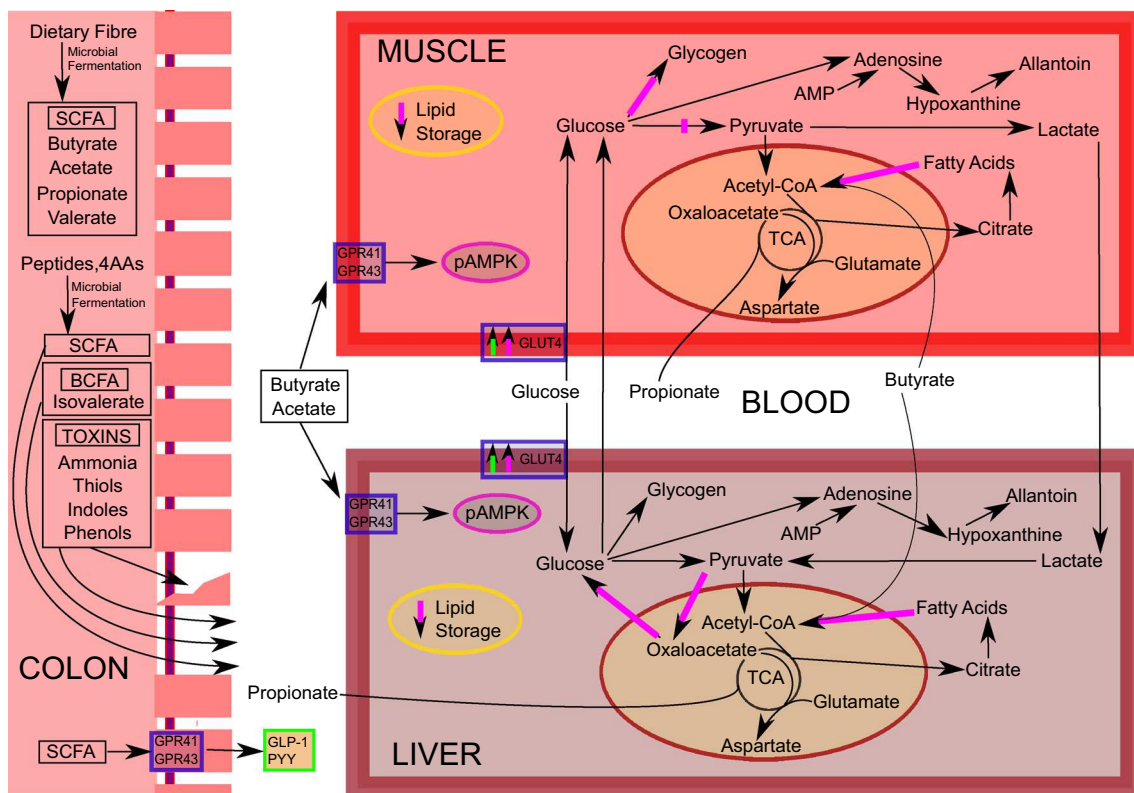
Fig. 4 Fermentation of indigestible foods in the colon results in the production of short-chain fatty acids (SCFA) by microbial fermentation. SCFA in the colon bind to GPR41/43, which may lead to the production of the gut hormones peptide YY (PYY) and glucagon-like peptide-1 (GLP-1) that enhance glucose uptake (Canfora et al. 2015). Acetate and butyrate in the blood bind to GPR41/43 on cells to increase cellular fatty acid oxidation, glucose uptake and glycogen storage via the increased activation of adenosine monophosphate-activated protein kinase (AMPK) to phosphorylated AMPK (pAMPK) and production of glucose transporter type 4 (GLUT4).

Propionate may directly contribute to gluconeogenesis in the liver via succinyl-CoA. Generally small amounts of propionate and butyrate enter the circulation but increased concentration of SCFA in the colon or increased intestinal permeability will increase the amount of SCFA that enters. Intestinal permeability may be increased by the toxic byproducts of undigested peptides and amino acids that are fermented into SCFA and branched-chain fatty acids (BCFA). *Purple arrows* are actions by activated AMPK (pAMPK), *green arrows* are actions by GLP-1 and PYY

(Fig. 1a). Anaerobic bacteria appeared to largely contribute to this decrease. A number of anaerobic bacteria species appear to trend towards a decrease, but of these only the genus *Bacteroides* spp. (specifically Other *Bacteroides* species) was significant (Fig. 1a). *Bacteroides* species, other than B. *vulgatus* and B. *uniformis*, appear to be relevant to ME/CFS and further studies to elucidate the precise bacterial species is pertinent.

Total bacteria had the lowest *P* value (Fig. 1a) indicating that microbe content may be affected by fecal composition and density. To assess the proportion of bacteria independent of these factors, we divided each microbial count by the total bacteria in that sample, presented in Fig. 1a as relative count. The only significantly altered microbe in ME/CFS was an increase of *Clostridium* spp.

Using the same culturing methods that we used previously, an increase in aerobic bacteria in ME/CFS has been observed (Sheedy et al. 2009). More recent research into gut dysbiosis in ME/CFS used 16S ribosomal RNA sequencing showing a decrease in bacterial species in ME/CFS, particularly for members of the Firmicutes phylum (Giloteaux et al. 2016). Both these studies have used different categorization of microbes, which makes comparison difficult; however, others have also observed that *Bacteroides* spp. were decreased (Fremont et al. 2013). Importantly a microbial difference between ME/CFS and non-ME/CFS control subjects is being observed across studies.

3.4 Significantly altered fecal metabolites in patients with ME/CFS

The absolute concentrations of three fecal metabolites from the ^{1}H NMR analysis were significantly different between the ME/CFS and non-ME/CFS cohorts (Fig. 1e). Valerate and isovalerate were increased and lactate was decreased in ME/CFS patients. When considering metabolites as a function of total metabolite concentrations (relative abundance data) it was found that valerate and isovalerate were again increased along with an increase of butyrate (Fig. 1e).

An unsupervised PCA analysis was conducted using fecal metabolites and a separation of ME/CFS patients from non-ME/CFS subjects was observed by the scores plot (PC1: 49.73% and PC2: 13.08%) in Fig. 1f. The loadings plot revealed that isovalerate and valerate are contributing significantly to the separation (Fig. 1f). Glutamine was grouped with isovalerate and valerate signifying a potential link, other metabolites contributing to separation, but not individually significant, were propionate, acetate, and succinate.

An overall increase of SCFA was observed in the ME/CFS cohort compared to the non-ME/CFS. Acetate, butyrate and propionate are the main three SCFA of the gut and are essential for gut health (Scheppach 1994; Wong et al. 2006) and all three trended to an increase with butyrate being statistically significant. Butyrate is a major fuel of colonocytes and its increase thought important for gut health (Canani et al. 2011). Isovalerate and valerate are two lesser researched SCFA, but both appear to be important to ME/CFS. Isovalerate is primarily produced from the fermentation of peptides and amino acids (Cardona et al. 2005), namely leucine (Zarling and Ruchim 1987) while valerate is produced by fermentation of either carbohydrates or peptides, similarly to acetate, butyrate and propionate, but in much smaller quantities and dependent on a select group of bacteria (Bourriaud et al. 2005). Lactate has been shown to be fermented into SCFA by bacteria within the gut (Bourriaud et al. 2005).

The metabolite changes we observed in ME/CFS indicate an increase in fermentation, a process that may occur due to a higher pH, a decreased gut transit or a change in the bacterial species of the microbiota. Studies on pH in the gut have concluded that as it becomes more alkaline the concentration of lactate decreases and its fermentation to SCFA increases (Belenguer et al. 2007). Amino acids and peptides are also increasingly fermented to SCFA under alkaline conditions (Rasmussen et al. 1988; Smith and Macfarlane 1998). Levels of all amino acids (except glutamine) were decreased in ME/CFS patients compared to controls although none were significant (Fig. 1e). Valerate and isovalerate production from amino acids and peptides has been shown to increase significantly with pH (Walker et al. 2005). Both isovalerate and valerate grouped with glutamine during PCA analysis (Fig. 1f) suggesting that amino acids may be transferring their amino group to produce glutamine when fermented in the colon of ME/CFS patients. The microbial data also appears consistent with an increased fermentation in ME/CFS as *Clostridium* spp. was observed to be increased in ME/CFS patients. *Clostridium* species are known to make butyrate from lactate and amino acids to produce SCFA (Smith and Macfarlane 1998). Intestinal permeability is suspected in ME/CFS patients (Giloteaux et al. 2016) and the byproducts of fermenting amino acids may enhance this (Fig. 4).

Previous findings of microbial alterations in ME/CFS suggested an increase in lactic acid producing bacteria (Sheedy et al. 2009). We therefore expected an increase in lactate and a decrease in SCFA as observed in the feces of IBD patients (Huda-Faujan et al. 2010); however, we observed the opposite. Although SCFA are considered to have positive health benefits their increase has been observed in autism spectrum disorder (ASD) (Wang et al. 2012). Intraventricular additions of propionate and SCFA have been proposed to induce ASD in developing rats with biochemical features of mitochondrial dysfunction, increased oxidative stress with glutathione depletion, altered lipid profiles and an innate neuroinflammatory

response (Macfabe 2012). These symptoms of increased propionate and SCFA in the blood are similar to current findings from research on ME/CFS (Armstrong et al. 2014; Maes et al. 2011; Naviaux et al. 2016). It is possible that the previously proposed increase in intestinal permeability (Fremont et al. 2013; Giloteaux et al. 2016) along with the increase in SCFA observed here are producing a significant influx of SCFA, especially propionate, into the bloodstream that may be causing or exacerbating mitochondrial dysfunction and oxidative stress in ME/CFS. To extract more information from our dataset we combined the fecal bacterial count and fecal metabolite concentrations with serum and urine metabolite concentrations to enable visualization of the gut-host interactions and may give information regarding how the increased SCFA may be affecting ME/CFS.

3.5 ME/CFS correlations of fecal, blood serum and urine metabolites

From ^{1}H NMR analysis 29 metabolites were quantitated and identified in the blood sera and 30 in the urine (Armstrong et al. 2015). The significant metabolites from the blood serum and urine are presented in Fig. 1b. In the serum and urine analysis we surmised a reduced use of the TCA cycle due to a decreased glycolysis (or increased gluconeogenesis) increasing the usage of amino acids for the production of ATP. As depicted in Fig. 4, SCFA are known to influence the host energy metabolism by increasing fatty acid oxidation and gluconeogenesis (den Besten et al. 2013b), with the latter being observed in the non-ME/CFS cohort by fecal SCFA negatively correlating with serum and urine gluconeogenesis substrates (Figs. 2, 3). However, no correlation between fecal SCFA and gluconeogenesis substrates in ME/CFS patients was observed.

The urine and blood metabolite anomalies suggested an increased use of amino acids to produce ATP, an increased rate of gluconeogenesis (or decreased rate of glycolysis), and a reduced level of purine synthesis substrates and products (Armstrong et al. 2015). The metabolites significant to these pathways in the blood serum positively correlated with fecal amino acids in ME/CFS patients, suggesting that reduced amino acids in the feces are related to the exacerbation of these pathways. This anomaly may reflect reduced amino acid availability in the gut leading to a depletion of amino acids as substrates for these altered host pathways in ME/CFS patients. Additionally, fecal amino acids may be decreased due to increased fermentation to produce SCFA in ME/CFS, which may enhance the rate of gluconeogenesis. However, no direct relationship between SCFA and the altered host metabolism pathways was observed and the nature of this relationship is yet to be determined, it is

plausible that the metabolite anomalies of either biofluid may affect the other.

In the absolute dataset almost all fecal metabolites positively correlated with one another in the non-ME/CFS cohort while in the ME/CFS cohort there was no correlation observed between fecal amino acids and fecal SCFA (Fig. 2). The lack of correlation in ME/CFS between SCFA and amino acids within the fecal biofluid may be due to a transport issue, multiple factors of fermentation and/or variance within the ME/CFS cohort (Fig. 1e).

The relative data (Fig. 3) highlighted that propionate and butyrate positively correlated with other SCFA in the non-ME/CFS cohort but not the ME/CFS group. The lack of this correlation in the ME/CFS cohort suggests that the SCFA increase from fermentation may be due to a disturbance of the gut microbiome. In the absolute dataset the correlation between fecal butyrate and serum citrate was positive in ME/CFS and negative in non-ME/CFS. Likewise, this same correlation occurred in the relative dataset suggesting it may be a key differentiation between the two groups. SCFAs trigger the oxidation of free fatty acids which reduces lipid synthesis (Canfora et al. 2015), citrate is the beginning of lipid synthesis and the non-ME/CFS cohort data aligns with this. In healthy individuals, minimal amounts of butyrate are expected to enter the blood to produce acetyl-CoA and potentially citrate; however, in ME/CFS an increased intestinal permeability (Sheedy et al. 2009) may increase the levels of butyrate entering the blood, which would explain the relationship we observe here. Serum citrate was also observed to negatively correlate with fecal glycerol, isoleucine, leucine, and phenylalanine, which breakdown to form ketones that produce acetyl-CoA in mitochondria. Citrate has been shown previously to negatively correlate with ketone bodies (Baticz et al. 2002). Furthermore, several of these fecal amino acids are positively correlated to serum hypoxanthine and urine trigonelline, breakdown products of ATP and niacin (a constituent of NADH) respectively. An increase of citrate, a decrease of ATP, a decrease of NADH and the increased use of amino acids for energy metabolism have all been observed when aconitase has been inhibited by ROS (Tretter and Adam-Vizi 2000). The proposed oxidative-stress induced dysfunction of mitochondria via aconitase inhibition in ME/CFS may result in increased citrate production from ketogenic amino acids and butyrate (Vasquez-Vivar et al. 2000; Yamano et al. 2016).

3.6 ME/CFS correlations of fecal microbes with fecal, blood serum and urine metabolites

Fecal metabolite changes between ME/CFS and non-ME/CFS suggest increased SCFA from fermentation from amino acids in ME/CFS patients with links to reduced

serum metabolites previously found to be significant. Microbe differences were also observed, with decreases of *Bacteroides* spp. and increases of *Clostridium* spp. in ME/CFS patients. The correlation of fecal metabolites with serum and urine metabolites varied between ME/CFS and non-ME/CFS, indicating that perhaps multiple factors within the gut separate these two groups. By observing the correlation of microbes to metabolites across all three biofluids we can determine any associations that microbes may have with the differences observed in metabolites.

Significantly reduced serum metabolites that positively correlated with fecal amino acids in ME/CFS patients also positively correlated with *Bacteroides* spp. and *B. uniformis* in absolute data. *Streptococcus* spp., Other *Streptococcus* spp. and Yeast were also positively correlated with many of these serum metabolites as well as positively correlating with fecal amino acids. The relevance of these microbes to fecal amino acids is difficult to determine, it may suggest that one is influencing the other or that both are influenced by the gut environment. Other *Streptococcus* spp. was the only bacteria group to negatively correlate with urine amino acids in the non-ME/CFS cohort that mirrored the positive effects of SCFA in reducing metabolite excretion.

In the absolute data (Fig. 2) there were positive correlations between *B. vulgatus, B. uniformis, Bacteroides* spp., anaerobic bacteria, and total bacteria. Unique to ME/CFS is that Other *Bacteroides* spp. positively correlate with *Bacteroides* spp., anaerobic bacteria and total bacteria along with aerobic bacteria, Coliform bacteria and *E. coli*, suggesting that *Bacteroides* spp. may be a central microbial difference between ME/CFS and non-ME/CFS.

Valerate and isovalerate positively correlated with each other in both cohorts and both were significantly increased in ME/CFS (Fig. 1b). In the relative dataset isovalerate and valerate positively correlated with aerobic bacteria, Coliform bacteria and *E. coli*, and negatively correlated with anaerobic bacteria in ME/CFS only. The increased proportion of valerate and isovalerate seems to relate to the increased ratio of aerobes to anaerobes, which has been observed previously in ME/CFS (Giloteaux et al. 2016; Sheedy et al. 2009). These were the only correlations observed between SCFA and microbes in ME/CFS, the increased fecal butyrate and increased *Clostridium* spp. in ME/CFS did not positively correlate. This lack of correlation needs to be investigated further as metabolite differences between the cohorts suggested expected links that were observed in the non-ME/CFS correlation but not in the ME/CFS correlations. Correlations between biofluids in both groups appeared significantly altered and underlying differences might be significant to the disorder and the lack of correlation in ME/CFS may represent significant variations within the ME/CFS cohort.

4 Conclusions

The workflow presented produced new information on the relationship of fecal metabolites and microbes with host blood serum and urine metabolites in ME/CFS. It is important to note that although correlations do not produce cause-effect results, the relationship of these variables allows for a basis for specific studies to observe the path of cause and effect. As expected in the non-ME/CFS cohort fecal SCFA negatively correlated with most metabolites in the serum and urine and increased production of SCFA in the gut by microbes associates with increased gluconeogenesis and reduced lipogenesis (Fig. 4).

In the ME/CFS cohort, *Bacteroides* spp. decreased and *Clostridium* increased while fecal butyrate, valerate and isovalerate significantly increased and lactate decreased. These results were consistent with increased microbial fermentation at the expense of amino acids in the gut of ME/CFS patients. Byproducts of amino acid fermentation may enhance gut epithelium permeability (Canfora et al. 2015) and we propose that an increased influx of larger than normal concentrations of SCFA into the blood may have deleterious effects on ME/CFS patients. Correlational analysis on the ME/CFS cohort provided a positive correlation of fecal amino acids with previously reported blood and urine metabolite anomalies in ME/CFS (Armstrong et al. 2015).

Further research into the role of the gut microbiome and metabolites in ME/CFS pathogenesis is necessary, larger cohorts and longitudinal studies using a similar workflow as presented here are encouraged to replicate and further elucidate these findings.

Compliance with ethical standards

Conflict of interest   There were no conflicts of interest.

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