The association of fecal microbiota and fecal, blood serum and urine metabolites in Myalgic Encephalomyelitis/Chronic Fatigue Syndrome

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27

28 **Data Deposition**

29 The data for this study has been deposited at MetaboLights
30 (http://www.ebi.ac.uk/metabolights) with accession number MTBLS369.

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. .

53 **Keywords**

54 Myalgic Encephalomyelitis/Chronic Fatigue Syndrome; Feces; Microbiota; Short chain fatty

55 acids; Energy metabolism; Amino acids

56

57 **Abbreviations**

58 BCFA - Branched-chain fatty acids

59 IBD – Irritable Bowel Disease

60 ME/CFS - Myalgic Encephalomyelitis/Chronic Fatigue Syndrome

61 NOESY – Nuclear overhauser effect spectroscopy

62 NMR - Nuclear Magnetic Resonance

63 PCA – Principal component analysis

64 SCFA - Short chain fatty acids

65 TOCSY – Total correlated spectroscopy

66 **1 Introduction**

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

76 Recent research has been conducted on the impact of diet and the gut microbiota on
77 metabolite production and its relevance to disease (Guarner, Malagelada 2003).
78 Understanding the symbiotic relationships of host-microbe and microbe-microbe interactions
79 is complicated as these relationships are altered by reactions to each other making it difficult
80 to deduce an origin of cause and the subsequent effect. In this respect, the detailed systems
81 biology understanding of the impact of individual microbes upon the gut and the host and
82 how they are cultivated remains to be elucidated. To determine the impact of microbes on
83 health, metabolomics offers a practical approach to investigate the host-microbe relationships
84 through the correlations of identified and quantitated bacteria with host metabolites (Li,
85 Wang, Zhang, Rantalainen, Wang, Zhou, Zhang et al. 2008; Schaubeck, Clavel, Calasan,
86 Lagkouvardos, Haange, Jehmlich, Basic et al. 2016). The impact of microbes on host health
87 is already being realized through fecal transplant treatments which effectively transplant the
88 diseased "hidden organ" with a healthy donor microbiome. This treatment has been claimed
89 to be successful for treating a number of illnesses including ulcerative colitis (Moayyedi,
90 Surette, Kim, Libertucci, Wolfe, Onischi, Armstrong et al. 2015), Metabolic Syndrome

91 (Vrieze, Van Nood, Holleman, Salojarvi, Kootte, Bartelsman, Dallinga-Thie et al. 2012),
92 Multiple Sclerosis (Borody, Leis, Campbell, Torres, Nowak 2011) and Myalgic
93 Encephalomyelitis/Chronic Fatigue Syndrome (ME/CFS) (Borody, Brandt, Paramsothy 2014;
94 Borody, Khoruts 2012).

95 ME/CFS is a long-term disorder of fatigue that is exacerbated by exercise and not
96 sufficiently relieved by rest. The fatigue is accompanied by a host of other symptoms,
97 including post-exertional malaise and various symptoms of cognitive dysfunction, chronic
98 immune activation, neuroendocrine dysregulation, heightened sensitivity, cardiovascular
99 issues and gut dysbiosis. The disorder is poorly understood, the mechanism of its
100 pathogenesis is likely to be multi-faceted including predisposing, triggering, and maintenance
101 factors (White 2004). The history of research into ME/CFS is littered with findings from
102 studies that have subsequently not been replicated. However, recent metabolomics studies on
103 blood in combination with past metabolite studies have produced a set of consistent factors
104 including: increased oxidative stress, depleted amino acids and lipids, depleted purine
105 metabolites, reduced folate cycle, increased sugars and depletion of TCA cycle metabolites
106 (Armstrong, McGregor, Lewis, Butt, Gooley 2015; Armstrong, McGregor, Sheedy, Buttfield,
107 Butt, Gooley 2012; Georgiades, Behan, Kilduff, Hadjicharalambous, Mackie, Wilson, Ward
108 et al. 2003; Jacobson, Saich, Borysiewicz, Behan, Behan, Wreghitt 1993; McGregor,
109 Dunstan, Zerbes, Butt, Roberts, Klineberg 1996; Naviaux, Naviaux, Li, Bright, Alaynick,
110 Wang, Baxter et al. 2016; Niblett, King, Dunstan, Clifton-Bligh, Hoskin, Roberts, Fulcher et
111 al. 2007; Pall 2002; Suarez, Guillamo, Roig, Blazquez, Alegre, Bermudez, Ventura et al.
112 2010; Yamano, Sugimoto, Hirayama, Kume, Yamato, Jin, Tajima et al. 2016). Along with
113 increased oxidative stress it has been observed that there are immune anomalies consistent
114 with altered immune activation (Hornig, Montoya, Klimas, Levine, Felsenstein, Bateman,
115 Peterson 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, Goodrich, Walters, Levine, Ley, Hanson 2016; Sheedy, Wettenhall, Scanlon, Gooley, Lewis, McGregor, Stapleton 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, Jain, De Meirleir, Peterson, Klimas, Lerner, Bested 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

140 study was approved by the University of Melbourne human research ethics committee
141 (HREC# 0723086).
142 All subjects were asked to list their current medications and oral supplements. Prior
143 to fecal sample collection, patients were instructed to cease antibiotic and/or probiotic
144 treatment for four and two weeks, respectively. Patients collected a sample of their first
145 morning bowel motion in a fecal container (anaerobic pouch system) with a perforated lid to
146 aid anaerobiosis (achieved by activating Anaero Gen Compact (Oxoid, Thermo Fisher
147 Scientific, Australia)). Samples were immediately transported to the laboratory in cold
148 conditions (<12 °C) for analysis within 48 h after collection. Laboratory protocol rejects
149 samples subjected to inaccurate collection, transportation, anaerobiosis or refrigeration
150 procedures. Internal quality assurance investigations validated the anaerobic transport and
151 culture methods. After removal from the anaerobic pouch system, all fecal samples were
152 processed within 10-15 min.
153

154 **2.2 Sample Preparation for NMR Analysis**

155 A small portion of each fecal sample was homogenized by vortex in deuterated saline
156 at 1:10 w/v before being syringe filtered (0.2 µm, 25 mm diameter) and dispensed into five 1
157 mL aliquots for storage at -80 ºC. All fecal solution samples were prepared for NMR analysis
158 using a liquid-liquid extraction technique (Sheedy, Ebeling, Gooley, McConville 2010). All
159 59 fecal solution samples were prepared simultaneously. A 250 µL sample of each fecal
160 solution was added to 250 µL ice-cold deuterated chloroform and 250 µL ice-cold deuterated
161 methanol and mixed by vortexing before being left to sit on ice for 15 min. Samples were
162 then centrifuged (16,100 $\times$ *g)* at 4 ºC for 10 min to produce a biphasic mixture with a
163 hydrophilic phase of water/deuterated methanol and lipophilic phase of deuterated
164 chloroform. A 297 µL sample of the top hydrophilic layer was added to an equal volume of

165 200 mM sodium phosphate in ^2H_2O (pH 7) and 66 μL of ^2H_2O containing 5 mM DSS and
166 0.2% (w/v) sodium azide to make a total volume of 660 μL. The sample was then mixed by
167 vortex, centrifuged for 1 min at 13,000 rpm and 550 μL of supernatant was transferred to a 7-
168 inch 5-mm 507-grade NMR tube for NMR analysis. While the apolar layer was discarded,
169 future studies are intended to investigate the lipid profiles from serum and fecal samples.
170
171 **2.3 Microbial Identification and Quantification**.
172 Between 0.5-1.0 g of fecal matter was transferred to 10 mL of 1% glucose-saline buffer
173 and dilutions from 10^{-1} to 10^{-7} of homogenised fecal samples were prepared (Willis 1991).
174 Dilutions (10 and/or 1 μL amounts) were transferred onto Columbia horse blood agar
175 (Oxoid), chromogenic medium (Oxoid), colistin and nalidixic acid blood selective agar
176 (Oxoid), and chloramphenicol-gentamicin selective Sabouraud agar for aerobic incubation.
177 Anaerobic incubation (four day duration) in anaerobic jars (Oxoid) utilised pre-reduced
178 Columbia horse blood haemin agar and Raka Ray medium. Aerobic media were incubated at
179 35 °C for 48 h. A stereomicroscope was used to examine both aerobic and anaerobic culture
180 plates for a minimum of 20 min/plate before bacterial identification. Each colony from each
181 medium was microscopically examined and the colony/viable count were quantified for each
182 plate. To assess microbial purity prior to identification, similar morphotypes were sub-
183 cultured onto horse blood agar.
184 Following overnight purity checks, index bacterial colonies were transferred to a target
185 polished steel plate (MSP 96, Bruker Daltonics Inc.) for drying at room temperature. Air-
186 dried samples were subjected to protein extraction with 1 μL 70% formic acid (Sigma). After
187 repeat air-drying under exhaust ventilation, samples were overlaid with 1 μL of matrix
188 solution (saturated solution of α-cyano-4-hydroxycinnamic acid (HCCA) in a mixture of
189 47.5% 18 mOhm water, 2.5% trifluoroacetic acid, and 50% acetonitrile). Dried samples were

190 analysed using Microflex MALDI-TOF mass spectrometer (Bruker Daltonik GmbH, Leipzig,
191 Germany) equipped with a 60 Hz nitrogen laser. Spectra were recorded in the positive linear
192 mode for the mass range of 2-20 kDa at maximum laser frequency. The MALDI Biotyper 3.0
193 software package (default settings; Daltonik GmbH, Bremen, Germany) was used to
194 automatically analyse and measure raw spectra without user intervention.

195

196 **2.4 NMR Measurements**

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

211

212 **2.5 Data Analysis**

213 The workflow used for analysis is displayed in Fig. 1 and is discussed in section 3.1. Prior to
214 statistical analysis all data were assessed for normality. Those metabolites or microbial

215 counts not found to be normally distributed were either log converted in the raw data set or
216 arcsine converted for the relatively distributed data set. T-tests and Benjamini-Hochberg
217 adjustments were conducted on the identified fecal metabolites and microbial counts to
218 establish significant metabolites and microbes. Correlation graphs were developed in-house
219 using Microsoft Excel 2010 to overlay the correlations within both the ME/CFS and non-
220 ME/CFS to highlight discrepancies.

221

222 **3 Results and Discussion**

223 **3.1 The Experimental Workflow**

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

230 Metabolite data analysis followed the same workflow for the three separate biofluids
231 but is only depicted in Fig. 1 for feces. A table of the significant metabolites from serum and
232 urine (Fig. 1b) are provided (Armstrong, McGregor, Lewis, Butt, Gooley 2015).
233 Unsupervised PCA analysis was conducted on the fecal ^{1}H NMR spectra by using spectral
234 'binned' data (Fig. 1c) to observe a separation of the ME/CFS and non-ME/CFS cohorts and
235 to highlight all peaks that contributed to that separation for metabolite identification and
236 quantitation. The identification and quantitation of fecal metabolites from peaks on the ^{1}H
237 NMR spectra (Fig. 1d) were conducted using Chenomx software. The statistical significance
238 of fecal metabolites between the ME/CFS and non-ME/CFS groups were analyzed using
239 Student T-tests and were presented along with fold change between the groups (Fig. 1e).

240 Student T-tests were also used to analyse the statistical significance of microbes (Fig. 1a) and
241 the serum and urine metabolites (Fig. 1b) between the ME/CFS and non-ME/CFS groups.
242 Microbes were categorized mostly by genus with Bacteroides spp. and Streptococcus
243 spp. further identified into several species (Fig. 1a). Bacteria were totaled and sub-totaled as
244 aerobic and anaerobic bacteria. The metabolite concentrations and microbial counts are
245 presented in both absolute (or raw) values and relative distribution (% of total) values where
246 the latter accounts for fecal, urine and blood serum dilution factors that vary from sample to
247 sample. Normalising all datasets to the same two data types enables Pearson correlation
248 analyses across all the metabolites and microbes in both absolute (Fig. 2) and relative (Fig. 3)
249 forms. Correlation coefficients (*r*) from Pearson correlation analysis quantitates the strength
250 of two separate factors fluctuating with one another from sample to sample across a whole
251 cohort. The relationship of microbes and fecal metabolites were studied with one another and
252 then with serum and urine metabolites (Fig. 2 and 3).
253 The correlational analyses in Fig. 2 and 3 allow us to observe the association of fecal
254 microbes and metabolites of serum, urine and feces in the two distinct cohorts of ME/CFS
255 and non-ME/CFS. Expectedly, most correlations occurred within the same biofluid; however,
256 there were also a large number of relationships existing in inter-biofluid correlations. Fig. 2
257 shows large discrepancies between the ME/CFS and non-ME/CFS with respect to fecal
258 amino acids and fecal organic acids. These differences are further defined when these fecal
259 metabolites are correlated to serum and urine metabolites. This disturbance in correlations
260 reflects the disparate biochemical signatures that exist between ME/CFS patients and non-
261 ME/CFS control groups as we observed 14 metabolites altered out of a total of 83 identified
262 metabolites (17%) across feces, urine and serum (Fig. 1b and 1e). These correlations offer
263 information on how these metabolite differences may be related to one another and other
264 metabolites across the host system. Information from these relationships depicted for

265 ME/CFS will be further extrapolated using previous research on metabolism and ME/CFS in
266 the following sections.
267

268 **3.2 Workflow evaluation through non-ME/CFS Control Correlations**

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

273 If we highlight the non-ME/CFS group on its own we see that fecal metabolites
274 positively correlate with one another in the absolute data (Fig. 2), indicating a fluctuation of
275 fecal metabolites depending on the water content and biological material in the feces. Most
276 fecal metabolites, particularly the short-chain fatty acids (SCFA), were negatively correlated
277 with total serum metabolites (Fig. 2). The effects of SCFA according to the literature appear
278 to be consistent here in these correlations of the non-ME/CFS control group (den Besten,
279 Lange, Havinga, van Dijk, Gerding, van Eunen, Muller et al. 2013; den Besten, van Eunen,
280 Groen, Venema, Reijngoud, Bakker 2013). Microbial SCFA are an important fuel source for
281 cells of the gut wall and also influence the energy metabolism of the host via their increased
282 incorporation into lipids in adipocytes and their increased oxidation to produce glucose and
283 glycogen in muscle cells and hepatocytes (Fig. 4) (Canfora, Jocken, Blaak 2015; den Besten,
284 van Eunen, Groen, Venema, Reijngoud, Bakker 2013). After removing the effect of
285 concentration of metabolites from the biofluids by observing the relative abundance (Fig. 3)
286 we still see that the SCFA, except for acetate, are negatively correlated to citrate. Propionate
287 enters the hepatocytes to enhance glucose production via gluconeogenesis (Fig. 4) and was
288 observed here to positively correlate with serum aspartate and urine glucose. These

289 observations suggest, as expected, that fecal SCFA entering the blood may be altering energy
290 metabolism by increasing gluconeogenesis and decreasing lipogenesis.

291 The bacteria *B. vulgatus* and *S. parasanguinis* positively correlated with most fecal
292 metabolites in the non-ME/CFS indicating that their concentrations are likely to be altered by
293 the volume of water and biological matter (Fig. 2). *S. parasanguinis* is prevalent within the
294 oral cavity and may be indicative of food consumption (Corby, Lyons-Weiler, Bretz, Hart,
295 Aas, Boumenna, Goss et al. 2005), while *B. vulgatus* is one of the most prevalent bacteria
296 found in feces (Rigottier-Gois, Rochet, Garrec, Suau, Dore 2003) and therefore may fluctuate
297 with the content of fecal material. Clostridium spp. and Bacteroides spp. are major fermenters
298 that produce SCFA (Van den Abbeele, Belzer, Goossens, Kleerebezem, De Vos, Thas, De
299 Weirdt et al. 2013), both as expected were observed to positively correlate with SCFA (Fig.
300 2).

301

302 **3.3 Significantly altered fecal microbes in patients with ME/CFS**

303 Student T-tests on the absolute count of microbes observed a significant decrease in
304 total bacteria in ME/CFS patients (Fig. 1a). Anaerobic bacteria appeared to largely contribute
305 to this decrease. A number of anaerobic bacteria species appear to trend towards a decrease,
306 but of these only the genus Bacteroides spp. (specifically Other Bacteroides species) was
307 significant (Fig. 1a). Bacteroides species, other than B. *vulgatus* and B. *uniformis*, appear to
308 be relevant to ME/CFS and further studies to elucidate the precise bacterial species is
309 pertinent.

310 Total bacteria had the lowest *p*-value (Fig. 1a) indicating that microbe content may be
311 affected by fecal composition and density. To assess the proportion of bacteria independent
312 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, Wettenhall, Scanlon, Gooley, Lewis, McGregor, Stapleton 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, Goodrich, Walters, Levine, Ley, Hanson 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, Coomans, Massart, De Meirleir 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

337 isovalerate and valerate signifying a potential link, other metabolites contributing to
338 separation, but not individually significant, were propionate, acetate, and succinate.

339 An overall increase of SCFA was observed in the ME/CFS cohort compared to the
340 non-ME/CFS. Acetate, butyrate and propionate are the main three SCFA of the gut and are
341 essential for gut health (Scheppach 1994; Wong, de Souza, Kendall, Emam, Jenkins 2006)
342 and all three trended to an increase with butyrate being statistically significant. Butyrate is a
343 major fuel of colonocytes and its increase thought important for gut health (Canani,
344 Costanzo, Leone, Pedata, Meli, Calignano 2011). Isovalerate and valerate are two lesser
345 researched SCFA, but both appear to be important to ME/CFS. Isovalerate is primarily
346 produced from the fermentation of peptides and amino acids (Cardona, Collinder, Stern,
347 Tjellstrom, Norin, Midtvedt 2005), namely leucine (Zarling, Ruchim 1987) while valerate is
348 produced by fermentation of either carbohydrates or peptides, similarly to acetate, butyrate
349 and propionate, but in much smaller quantities and dependent on a select group of bacteria
350 (Bourriaud, Robins, Martin, Kozlowski, Tenailleau, Cherbut, Michel 2005). Lactate has been
351 shown to be fermented into SCFA by bacteria within the gut (Bourriaud, Robins, Martin,
352 Kozlowski, Tenailleau, Cherbut, Michel 2005).

353 The metabolite changes we observed in ME/CFS indicate an increase in fermentation,
354 a process that may occur due to a higher pH, a decreased gut transit or a change in the
355 bacterial species of the microbiota. Studies on pH in the gut have concluded that as it
356 becomes more alkaline the concentration of lactate decreases and its fermentation to SCFA
357 increases (Belenguer, Duncan, Holtrop, Anderson, Lobley, Flint 2007). Amino acids and
358 peptides are also increasingly fermented to SCFA under alkaline conditions (Rasmussen,
359 Holtug, Mortensen 1988; Smith, Macfarlane 1998). Levels of all amino acids (except
360 glutamine) were decreased in ME/CFS patients compared to controls although none were
361 significant (Fig. 1e). Valerate and isovalerate production from amino acids and peptides has

362 been shown to increase significantly with pH (Walker, Duncan, McWilliam Leitch, Child,
363 Flint 2005). Both isovalerate and valerate grouped with glutamine during PCA analysis (Fig.
364 1f) suggesting that amino acids may be transferring their amino group to produce glutamine
365 when fermented in the colon of ME/CFS patients. The microbial data also appears consistent
366 with an increased fermentation in ME/CFS as Clostridium spp. was observed to be increased
367 in ME/CFS patients. Clostridium species are known to make butyrate from lactate and amino
368 acids to produce SCFA (Smith, Macfarlane 1998). Intestinal permeability is suspected in
369 ME/CFS patients (Giloteaux, Goodrich, Walters, Levine, Ley, Hanson 2016) and the
370 byproducts of fermenting amino acids may enhance this (Fig. 4).

371 Previous findings of microbial alterations in ME/CFS suggested an increase in lactic
372 acid producing bacteria (Sheedy, Wettenhall, Scanlon, Gooley, Lewis, McGregor, Stapleton
373 et al. 2009). We therefore expected an increase in lactate and a decrease in SCFA as observed
374 in the feces of IBD patients (Huda-Faujan, Abdulamir, Fatimah, Anas, Shuhaimi, Yazid,
375 Loong 2010); however, we observed the opposite. Although SCFA are considered to have
376 positive health benefits their increase has been observed in autism spectrum disorder (ASD)
377 (Wang, Christophersen, Sorich, Gerber, Angley, Conlon 2012). Intraventricular additions of
378 propionate and SCFA have been proposed to induce ASD in developing rats with
379 biochemical features of mitochondrial dysfunction, increased oxidative stress with
380 glutathione depletion, altered lipid profiles and an innate neuroinflammatory response
381 (Macfabe 2012). These symptoms of increased propionate and SCFA in the blood are similar
382 to current findings from research on ME/CFS (Armstrong, McGregor, Butt, Gooley 2014;
383 Maes, Kubera, Uytterhoeven, Vrydags, Bosmans 2011; Naviaux, Naviaux, Li, Bright,
384 Alaynick, Wang, Baxter et al. 2016). It is possible that the previously proposed increase in
385 intestinal permeability (Fremont, Coomans, Massart, De Meirleir 2013; Giloteaux, Goodrich,
386 Walters, Levine, Ley, Hanson 2016) along with the increase in SCFA observed here are

387 producing a significant influx of SCFA, especially propionate, into the bloodstream that may
388 be causing or exacerbating mitochondrial dysfunction and oxidative stress in ME/CFS. To
389 extract more information from our dataset we combined the fecal bacterial count and fecal
390 metabolite concentrations with serum and urine metabolite concentrations to enable
391 visualization of the gut-host interactions and may give information regarding how the
392 increased SCFA may be affecting ME/CFS.

393

394 **3.5 ME/CFS Correlations of Fecal, Blood serum and Urine Metabolites.**

395 From ^{1}H NMR analysis 29 metabolites were quantitated and identified in the blood sera and
396 30 in the urine (Armstrong, McGregor, Lewis, Butt, Gooley 2015). The significant
397 metabolites from the blood serum and urine are presented in Fig. 1b. In the serum and urine
398 analysis we surmised a reduced use of the TCA cycle due to a decreased glycolysis (or
399 increased gluconeogenesis) increasing the usage of amino acids for the production of ATP.
400 As depicted in Fig. 4, SCFA are known to influence the host energy metabolism by
401 increasing fatty acid oxidation and gluconeogenesis (den Besten, van Eunen, Groen, Venema,
402 Reijngoud, Bakker 2013), with the latter being observed in the non-ME/CFS cohort by fecal
403 SCFA negatively correlating with serum and urine gluconeogenesis substrates (Fig. 2 and 3).
404 However, no correlation between fecal SCFA and gluconeogenesis substrates in ME/CFS
405 patients was observed.

406 The urine and blood metabolite anomalies suggested an increased use of amino acids
407 to produce ATP, an increased rate of gluconeogenesis (or decreased rate of glycolysis), and a
408 reduced level of purine synthesis substrates and products (Armstrong, McGregor, Lewis,
409 Butt, Gooley 2015). The metabolites significant to these pathways in the blood serum
410 positively correlated with fecal amino acids in ME/CFS patients, suggesting that reduced
411 amino acids in the feces are related to the exacerbation of these pathways. This anomaly may

412 reflect reduced amino acid availability in the gut leading to a depletion of amino acids as
413 substrates for these altered host pathways in ME/CFS patients. Additionally, fecal amino
414 acids may be decreased due to increased fermentation to produce SCFA in ME/CFS, which
415 may enhance the rate of gluconeogenesis. However, no direct relationship between SCFA and
416 the altered host metabolism pathways was observed and the nature of this relationship is yet
417 to be determined, it is plausible that the metabolite anomalies of either biofluid may affect the
418 other.

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

424 The relative data (Fig. 3) highlighted that propionate and butyrate positively
425 correlated with other SCFA in the non-ME/CFS cohort but not the ME/CFS group. The lack
426 of this correlation in the ME/CFS cohort suggests that the SCFA increase from fermentation
427 may be due to a disturbance of the gut microbiome. In the absolute dataset the correlation
428 between fecal butyrate and serum citrate was positive in ME/CFS and negative in non-
429 ME/CFS. Likewise, this same correlation occurred in the relative dataset suggesting it may be
430 a key differentiation between the two groups. SCFAs trigger the oxidation of free fatty acids
431 which reduces lipid synthesis (Canfora, Jocken, Blaak 2015), citrate is the beginning of lipid
432 synthesis and the non-ME/CFS cohort data aligns with this. In healthy individuals, minimal
433 amounts of butyrate are expected to enter the blood to produce acetyl-CoA and potentially
434 citrate; however, in ME/CFS an increased intestinal permeability (Sheedy, Wettenhall,
435 Scanlon, Gooley, Lewis, McGregor, Stapleton et al. 2009) may increase the levels of butyrate
436 entering the blood, which would explain the relationship we observe here. Serum citrate was

437 also observed to negatively correlate with fecal glycerol, isoleucine, leucine, and
438 phenylalanine, which breakdown to form ketones that produce acetyl-CoA in mitochondria.
439 Citrate has been shown previously to negatively correlate with ketone bodies (Baticz,
440 Tomoskozi, Vida, Gaal 2002). Furthermore, several of these fecal amino acids are positively
441 correlated to serum hypoxanthine and urine trigonelline, breakdown products of ATP and
442 niacin (a constituent of NADH) respectively. An increase of citrate, a decrease of ATP, a
443 decrease of NADH and the increased use of amino acids for energy metabolism have all been
444 observed when aconitase has been inhibited by ROS (Tretter, Adam-Vizi 2000). The
445 proposed oxidative-stress induced dysfunction of mitochondria via aconitase inhibition in
446 ME/CFS may result in increased citrate production from ketogenic amino acids and butyrate
447 (Vasquez-Vivar, Kalyanaraman, Kennedy 2000; Yamano, Sugimoto, Hirayama, Kume,
448 Yamato, Jin, Tajima et al. 2016).
449

450 **3.6 ME/CFS Correlations of Fecal Microbes with Fecal, Blood Serum and Urine**
451 **Metabolites.**

452 Fecal metabolite changes between ME/CFS and non-ME/CFS suggest increased SCFA from
453 fermentation from amino acids in ME/CFS patients with links to reduced serum metabolites
454 previously found to be significant. Microbe differences were also observed, with decreases of
455 Bacteroides spp. and increases of Clostridium spp. in ME/CFS patients. The correlation of
456 fecal metabolites with serum and urine metabolites varied between ME/CFS and non-
457 ME/CFS, indicating that perhaps multiple factors within the gut separate these two groups.
458 By observing the correlation of microbes to metabolites across all three biofluids we can
459 determine any associations that microbes may have with the differences observed in
460 metabolites.

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

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

475 Valerate and isovalerate positively correlated with each other in both cohorts and both
476 were significantly increased in ME/CFS (Fig. 1b). In the relative dataset isovalerate and
477 valerate positively correlated with aerobic bacteria, Coliform bacteria and E. *coli,* and
478 negatively correlated with anaerobic bacteria in ME/CFS only. The increased proportion of
479 valerate and isovalerate seems to relate to the increased ratio of aerobes to anaerobes, which
480 has been observed previously in ME/CFS (Giloteaux, Goodrich, Walters, Levine, Ley,
481 Hanson 2016; Sheedy, Wettenhall, Scanlon, Gooley, Lewis, McGregor, Stapleton et al.
482 2009). These were the only correlations observed between SCFA and microbes in ME/CFS,
483 the increased fecal butyrate and increased Clostridium spp. in ME/CFS did not positively
484 correlate. This lack of correlation needs to be investigated further as metabolite differences
485 between the cohorts suggested expected links that were observed in the non-ME/CFS

486 correlation but not in the ME/CFS correlations. Correlations between biofluids in both groups
487 appeared significantly altered and underlying differences might be significant to the disorder
488 and the lack of correlation in ME/CFS may represent significant variations within the
489 ME/CFS cohort.
490

491 **4 Conclusions**

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

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

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

511 **Conflict of Interest Statement**

512 There were no conflicts of interest.

513

514 **Compliance with Ethical Requirements**

515 This study was approved by the University of Melbourne human research ethics committee

516 (HREC #0723086).

517

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521

522 **References**

523 Armstrong, C. W., N. R. McGregor, H. L. Butt, P. R. Gooley (2014). Metabolism in chronic fatigue
524 syndrome. Adv Clin Chem 66, 121-72
525 Armstrong, C. W., N. R. McGregor, D. P. Lewis, H. L. Butt, P. R. Gooley (2015). Metabolic profiling
526 reveals anomalous energy metabolism and oxidative stress pathways in chronic fatigue
527 syndrome patients. Metabolomics 11, 1626-1639 doi:10.1007/s11306-015-0816-5
528 Armstrong, C. W., N. R. McGregor, J. R. Sheedy, I. Buttfield, H. L. Butt, P. R. Gooley (2012). NMR
529 metabolic profiling of serum identifies amino acid disturbances in chronic fatigue syndrome.
530 Clin Chim Acta 413, 1525-31 doi:10.1016/j.cca.2012.06.022
531 Baticz, O., S. Tomoskozi, L. Vida, T. Gaal (2002). Relationship between concentration of citrate and
532 ketone bodies in cow's milk. Acta Vet Hung 50, 253-61 doi:10.1556/AVet.50.2002.3.1
533 Belenguer, A., S. H. Duncan, G. Holtrop, S. E. Anderson, G. E. Lobley, H. J. Flint (2007). Impact of pH
534 on lactate formation and utilization by human fecal microbial communities. Appl Environ
535 Microbiol 73, 6526-33 doi:10.1128/AEM.00508-07
536 Borody, T., S. Leis, J. Campbell, M. Torres, A. Nowak (2011). Fecal Microbiota Transplantation (FMT)
537 in Multiple Sclerosis (MS). American Journal of Gastroenterology 106, S352-S352
538 Borody, T. J., L. J. Brandt, S. Paramsothy (2014). Therapeutic faecal microbiota transplantation:
539 current status and future developments. Curr Opin Gastroenterol 30, 97-105
540 doi:10.1097/MOG.0000000000000027
541 Borody, T. J., A. Khoruts (2012). Fecal microbiota transplantation and emerging applications. Nat Rev
542 Gastroenterol Hepatol 9, 88-96 doi:10.1038/nrgastro.2011.244
543 Bourriaud, C., et al. (2005). Lactate is mainly fermented to butyrate by human intestinal microfloras
544 but inter-individual variation is evident. J Appl Microbiol 99, 201-12 doi:10.1111/j.1365-
545 2672.2005.02605.x

546 Canani, R. B., M. D. Costanzo, L. Leone, M. Pedata, R. Meli, A. Calignano (2011). Potential beneficial
547 effects of butyrate in intestinal and extraintestinal diseases. World J Gastroenterol 17, 1519-
548 28 doi:10.3748/wjg.v17.i12. 1519
549 Canfora, E. E., J. W. Jocken, E. E. Blaak (2015). Short-chain fatty acids in control of body weight and
550 insulin sensitivity. Nat Rev Endocrinol 11, 577-91 doi:10.1038/nrendo.2015.128
551 Cardona, M. E., E. Collinder, S. Stern, B. Tjellstrom, E. Norin, T. Midtvedt (2005). Correlation between
552 faecal iso-butyric and iso-valeric acids in different species. Microbial Ecology in Health and
553 Disease 17, 177-182
554 Carruthers, B. M., et al. (2003). Myalgic Encephalomyelitis/Chronic Fatigue Syndrome: Clinical
555 Working Case Definition, Diagnostic and Treatment Protocols. J Chronic Fatigue Syndrome
556 11, 7-36
557 Corby, P. M., et al. (2005). Microbial risk indicators of early childhood caries. J Clin Microbiol 43,
558 5753-9 doi:10.1128/JCM.43.11.5753-5759.2005
559 den Besten, G., et al. (2013). Gut-derived short-chain fatty acids are vividly assimilated into host
560 carbohydrates and lipids. Am J Physiol Gastrointest Liver Physiol 305, G900-10
561 doi:10.1152/ajpgi.00265.2013
562 den Besten, G., K. van Eunen, A. K. Groen, K. Venema, D. J. Reijngoud, B. M. Bakker (2013). The role
563 of short-chain fatty acids in the interplay between diet, gut microbiota, and host energy
564 metabolism. J Lipid Res 54, 2325-40 doi:10.1194/jlr.R036012
565 Dorrestein, P. C., S. K. Mazmanian, R. Knight (2014). Finding the missing links among metabolites,
566 microbes, and the host. Immunity 40, 824-32 doi:10.1016/j.immuni.2014.05.015
567 Fremont, M., D. Coomans, S. Massart, K. De Meirleir (2013). High-throughput 16S rRNA gene
568 sequencing reveals alterations of intestinal microbiota in myalgic encephalomyelitis/chronic
569 fatigue syndrome patients. Anaerobe 22, 50-6 doi:10.1016/j.anaerobe.2013.06.002
570 Georgiades, E., et al. (2003). Chronic fatigue syndrome: new evidence for a central fatigue disorder.
571 Clin Sci (Lond) 105, 213-8 doi:10.1042/CS20020354

572 Giloteaux, L., J. K. Goodrich, W. A. Walters, S. M. Levine, R. E. Ley, M. R. Hanson (2016). Reduced
573 diversity and altered composition of the gut microbiome in individuals with myalgic
574 encephalomyelitis/chronic fatigue syndrome. Microbiome 4, 30 doi:10.1186/s40168-016-
575 0171-4
576 Guarner, F., J. R. Malagelada (2003). Gut flora in health and disease. Lancet 361, 512-9
577 doi:10.1016/S0140-6736(03)12489-0
578 Guinane, C. M., P. D. Cotter (2013). Role of the gut microbiota in health and chronic gastrointestinal
579 disease: understanding a hidden metabolic organ. Therap Adv Gastroenterol 6, 295-308
580 doi:10.1177/1756283X13482996
581 Hornig, M., et al. (2015). Distinct plasma immune signatures in ME/CFS are present early in the
582 course of illness. Sci Adv 1, doi:10.1126/sciadv.1400121
583 Huda-Faujan, N., et al. (2010). The impact of the level of the intestinal short chain Fatty acids in
584 inflammatory bowel disease patients versus healthy subjects. Open Biochem J 4, 53-8
585 doi:10.2174/1874091X01004010053
586 Human Microbiome Project, C. (2012). Structure, function and diversity of the healthy human
587 microbiome. Nature 486, 207-14 doi:10.1038/nature11234
588 Jacobson, W., T. Saich, L. K. Borysiewicz, W. M. Behan, P. O. Behan, T. G. Wreghitt (1993). Serum
589 folate and chronic fatigue syndrome. Neurology 43, 2645-7
590 Li, M., et al. (2008). Symbiotic gut microbes modulate human metabolic phenotypes. Proc Natl Acad
591 Sci U S A 105, 2117-22 doi:10.1073/pnas.0712038105
592 Macfabe, D. F. (2012). Short-chain fatty acid fermentation products of the gut microbiome:
593 implications in autism spectrum disorders. Microb Ecol Health Dis 23,
594 doi:10.3402/mehd.v23i0.19260
595 Maes, M., M. Kubera, M. Uytterhoeven, N. Vrydags, E. Bosmans (2011). Increased plasma peroxides
596 as a marker of oxidative stress in myalgic encephalomyelitis/chronic fatigue syndrome
597 (ME/CFS). Med Sci Monit 17, SC11-5

598 McGregor, N. R., R. H. Dunstan, M. Zerbes, H. L. Butt, T. K. Roberts, I. J. Klineberg (1996). Preliminary
599 determination of a molecular basis of chronic fatigue syndrome. Biochem Mol Med 57, 73-
600 80
601 Moayyedi, P., et al. (2015). Fecal Microbiota Transplantation Induces Remission in Patients With
602 Active Ulcerative Colitis in a Randomized Controlled Trial. Gastroenterology 149, 102-109 e6
603 doi:10.1053/j.gastro.2015.04.001
604 Naviaux, R. K., et al. (2016). Metabolic features of chronic fatigue syndrome. Proc Natl Acad Sci U S A
605 113, E5472-80 doi:10.1073/pnas.1607571113
606 Niblett, S. H., et al. (2007). Hematologic and urinary excretion anomalies in patients with chronic
607 fatigue syndrome. Exp Biol Med (Maywood) 232, 1041-9 doi:10.3181/0702-RM-44
608 Nicholson, J. K., et al. (2012). Host-gut microbiota metabolic interactions. Science 336, 1262-7
609 doi:10.1126/science.1223813
610 Pall, M. L. (2002). Levels of Nitric Oxide Synthase Product Citrulline Are Elevated in Sera of Chronic
611 Fatigue Syndrome Patients. Journal of Chronic Fatigue Syndrome 10, 37-41
612 doi:10.1300/J092v10n03_04
613 Rasmussen, H. S., K. Holtug, P. B. Mortensen (1988). Degradation of Amino-Acids to Short-Chain
614 Fatty-Acids in Humans - an Invitro Study. Scandinavian Journal of Gastroenterology 23, 178-
615 182 doi:Doi 10.3109/00365528809103964
616 Rigottier-Gois, L., V. Rochet, N. Garrec, A. Suau, J. Dore (2003). Enumeration of Bacteroides species
617 in human faeces by fluorescent in situ hybridisation combined with flow cytometry using 16S
618 rRNA probes. Syst Appl Microbiol 26, 110-8 doi:10.1078/072320203322337399
619 Schaubeck, M., et al. (2016). Dysbiotic gut microbiota causes transmissible Crohn's disease-like ileitis
620 independent of failure in antimicrobial defence. Gut 65, 225-37 doi:10.1136/gutjnl-2015-
621 309333
622 Scheppach, W. (1994). Effects of short chain fatty acids on gut morphology and function. Gut 35,
623 S35-8

624 Sheedy, J. R., P. R. Ebeling, P. R. Gooley, M. J. McConville (2010). A sample preparation protocol for
625 1H nuclear magnetic resonance studies of water-soluble metabolites in blood and urine.
626 Anal Biochem 398, 263-5 doi:10.1016/j.ab.2009.11.027
627 Sheedy, J. R., et al. (2009). Increased d-lactic Acid intestinal bacteria in patients with chronic fatigue
628 syndrome. In Vivo 23, 621-8
629 Smith, E. A., G. T. Macfarlane (1998). Enumeration of amino acid fermenting bacteria in the human
630 large intestine: effects of pH and starch on peptide metabolism and dissimilation of amino
631 acids. Fems Microbiology Ecology 25, 355-368 doi:Doi 10.1016/S0168-6496(98)00004-X
632 Suarez, A., et al. (2010). Nitric oxide metabolite production during exercise in chronic fatigue
633 syndrome: a case-control study. J Womens Health (Larchmt) 19, 1073-7
634 doi:10.1089/jwh.2008.1255
635 Tretter, L., V. Adam-Vizi (2000). Inhibition of Krebs cycle enzymes by hydrogen peroxide: A key role
636 of [alpha]-ketoglutarate dehydrogenase in limiting NADH production under oxidative stress.
637 J Neurosci 20, 8972-9
638 Van den Abbeele, P., et al. (2013). Butyrate-producing Clostridium cluster XIVa species specifically
639 colonize mucins in an in vitro gut model. ISME J 7, 949-61 doi:10.1038/ismej.2012.158
640 Vasquez-Vivar, J., B. Kalyanaraman, M. C. Kennedy (2000). Mitochondrial aconitase is a source of
641 hydroxyl radical. An electron spin resonance investigation. J Biol Chem 275, 14064-9
642 Vrieze, A., et al. (2012). Transfer of intestinal microbiota from lean donors increases insulin
643 sensitivity in individuals with metabolic syndrome. Gastroenterology 143, 913-6 e7
644 doi:10.1053/j.gastro.2012.06.031
645 Walker, A. W., S. H. Duncan, E. C. McWilliam Leitch, M. W. Child, H. J. Flint (2005). pH and peptide
646 supply can radically alter bacterial populations and short-chain fatty acid ratios within
647 microbial communities from the human colon. Appl Environ Microbiol 71, 3692-700
648 doi:10.1128/AEM.71.7.3692-3700.2005

649 Wang, L., C. T. Christophersen, M. J. Sorich, J. P. Gerber, M. T. Angley, M. A. Conlon (2012). Elevated
650 fecal short chain fatty acid and ammonia concentrations in children with autism spectrum
651 disorder. Dig Dis Sci 57, 2096-102 doi:10.1007/s10620-012-2167-7
652 White, P. D. (2004). What causes chronic fatigue syndrome? BMJ 329, 928-9
653 doi:10.1136/bmj.329.7472.928
654 Willis, A. T. (1991). Anaerobic culture methods. In: Lovett, P. N. (ed) Anaerobic Microbiology A
655 Practical Approach The Practical Approach Series. .(pp1-12). IRL Press at Oxford University
656 Press, Oxford ; New York.
657 Wong, J. M., R. de Souza, C. W. Kendall, A. Emam, D. J. Jenkins (2006). Colonic health: fermentation
658 and short chain fatty acids. J Clin Gastroenterol 40, 235-43
659 Yamano, E., et al. (2016). Index markers of chronic fatigue syndrome with dysfunction of TCA and
660 urea cycles. Sci Rep 6, 34990 doi:10.1038/srep34990
661 Zarling, E. J., M. A. Ruchim (1987). Protein origin of the volatile fatty acids isobutyrate and
662 isovalerate in human stool. J Lab Clin Med 109, 566-70

663

664 **Figure Legends**

665 **Fig. 1** Workflow diagram to analyze fecal microbes and metabolites across blood serum,
666 urine and fecal samples. **a** The change and *P* value (*P*) of live microbe count identified in the
667 fecal samples of ME/CFS patients compared to non-ME/CFS subjects. **b** The significant
668 metabolites in blood serum and urine as fold change (FoldC) and *P* value (*P*) as previously
669 published (Armstrong, McGregor, Lewis, Butt, Gooley 2015). **c** The.PCA plot shows the
670 separation of the ME/CFS and non-ME/CFS using the area under the *curve* from 250 'bins'
671 (0.04 ppm wide) of the feces NMR spectra. **d** A 1D ^{1}H NMR spectrum of feces and its
672 metabolites, identified metabolites are annotated. **e** The fold change (FoldC) and *P*-value (*P*)
673 of metabolites identified within the feces of ME/CFS patients compared to non-ME/CFS
674 subjects. Metabolites were quantitated using Chenomx NMR suite 6.1. Data is presented in
675 both absolute and relative distribution forms. *P*-values have been adjusted using the
676 Benjamini-Hochberg equation and significant differences are highlighted in red. **f** PCA plots
677 using the relatively distributed blood serum metabolite data present a scores plot with
678 separation of the CFS and control cohorts and a loadings plot with the metabolites
679 distribution that contributed to the scores plot separation.

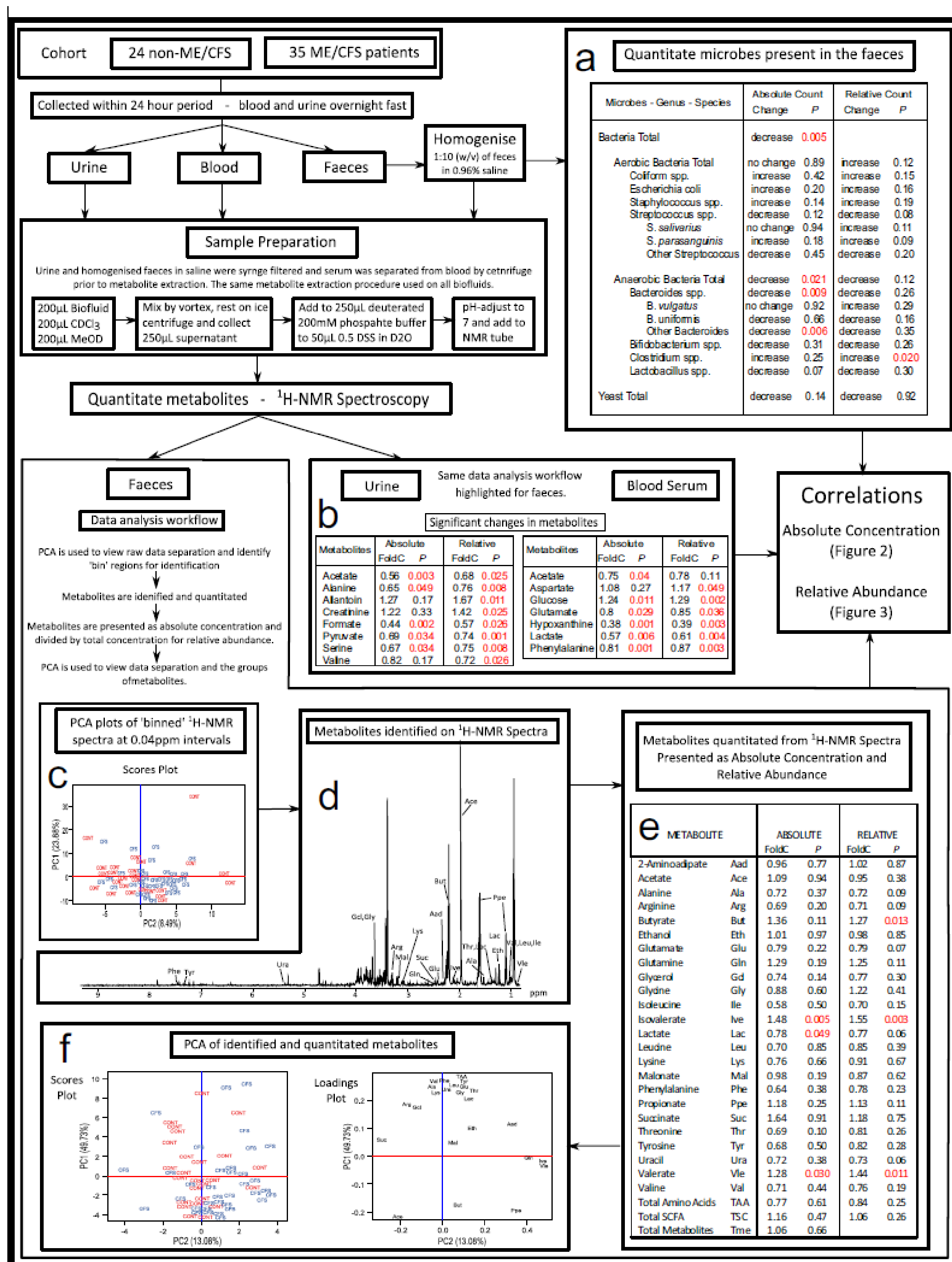
680

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

688 (blue, fluorescent blue, light blue) are a negative correlation in non-ME/CFS; and green hues
689 (green, light green) are no correlation in non-ME/CFS.
690
691 **Fig. 3** Relative abundance fecal metabolites and microbes correlated with all metabolites in
692 ME/CFS and non-ME/CFS cohorts. The correlations are displayed as colors in terms of
693 positive (r > 0.4), no correlation (-0.4 < r < 0.4), and negative (-0.4 > r) correlations in both
694 ME/CFS and non-ME/CFS groups. Dark-colors (red, green, blue) are a positive correlation in
695 ME/CFS; light-colors (light red, light green, light blue) are a negative correlation in ME/CFS;
696 and fluorescent-colors (fluorescent pink, fluorescent blue) are no correlation in ME/CFS. Red
697 hues (red, fluorescent pink, light red) are a positive correlation in non-ME/CFS; blue hues
698 (blue, fluorescent blue, light blue) are a negative correlation in non-ME/CFS; and green hues
699 (green, light green) are no correlation in non-ME/CFS.
700
701 **Fig. 4.** Fermentation of indigestible foods in the colon results in the production of short-chain
702 fatty acid (SCFA) by microbial fermentation. SCFA in the colon bind to GPR41/43, which
703 may lead to the production of the gut hormones peptide YY (PYY) and glucagon-like
704 peptide-1 (GLP-1) that enhance glucose uptake (Canfora, Jocken, Blaak 2015). Acetate and
705 butyrate in the blood bind to GPR41/43 on cells to increase cellular fatty acid oxidation,
706 glucose uptake and glycogen storage via the increased activation of AMPK (adenosine
707 monophosphate-activated protein kinase) to pAMPK (phosphorylated AMPK) and
708 production of glucose transporter type 4 (GLUT4). Propionate may directly contribute to
709 gluconeogenesis in the liver via succinyl-CoA. Generally small amounts of propionate
710 and butyrate enter the circulation but increased concentration of SCFA in the colon or
711 increased intestinal permeability will increase the amount of SCFA that enters. Intestinal
712 permeability may be increased by the toxic byproducts of undigested peptides and amino

713 acids that are fermented into SCFA and branched-chain fatty acids (BCFA). Purple arrows

714 are actions by activated AMPK (pAMPK), green arrows are actions by GLP-1 and PYY.



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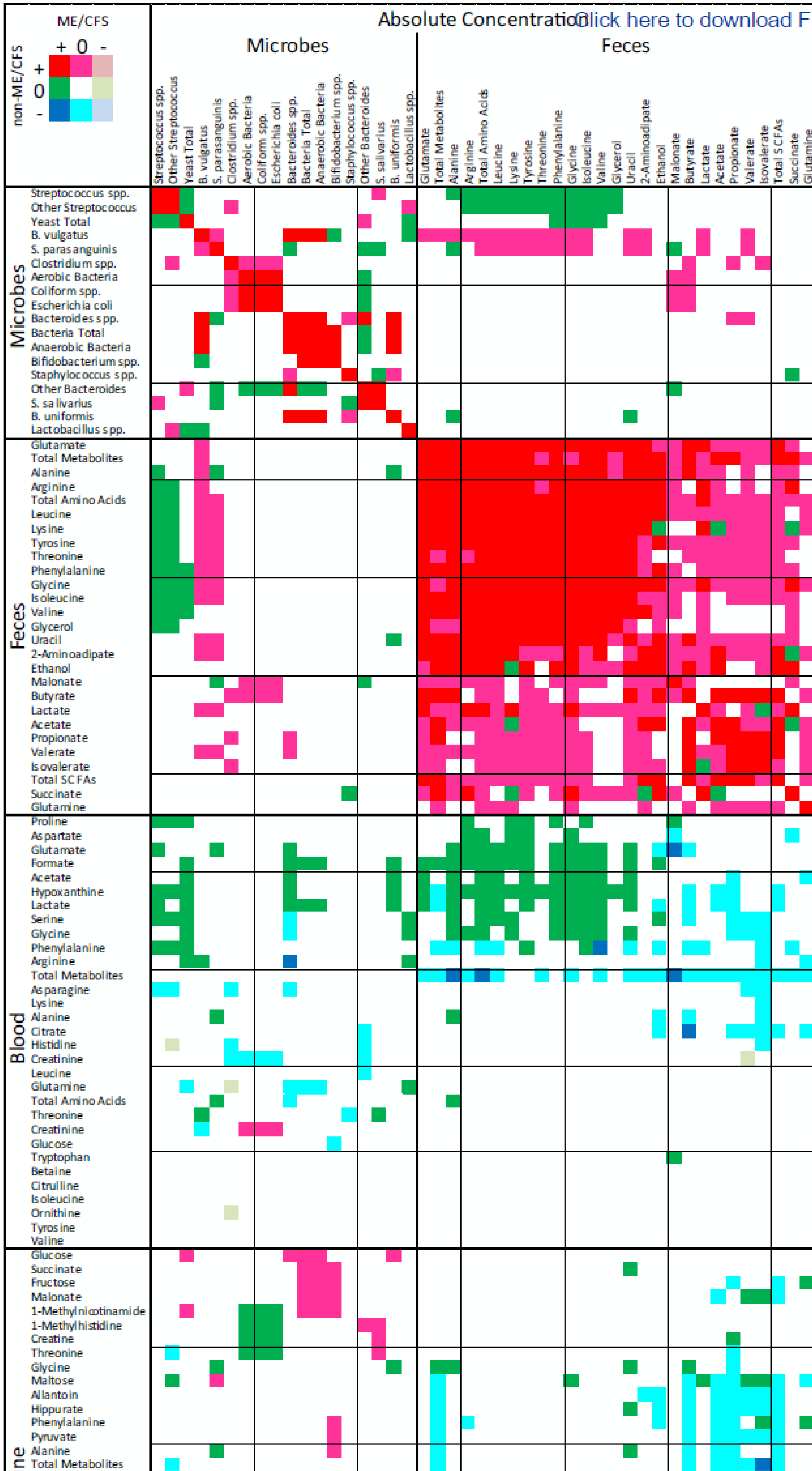

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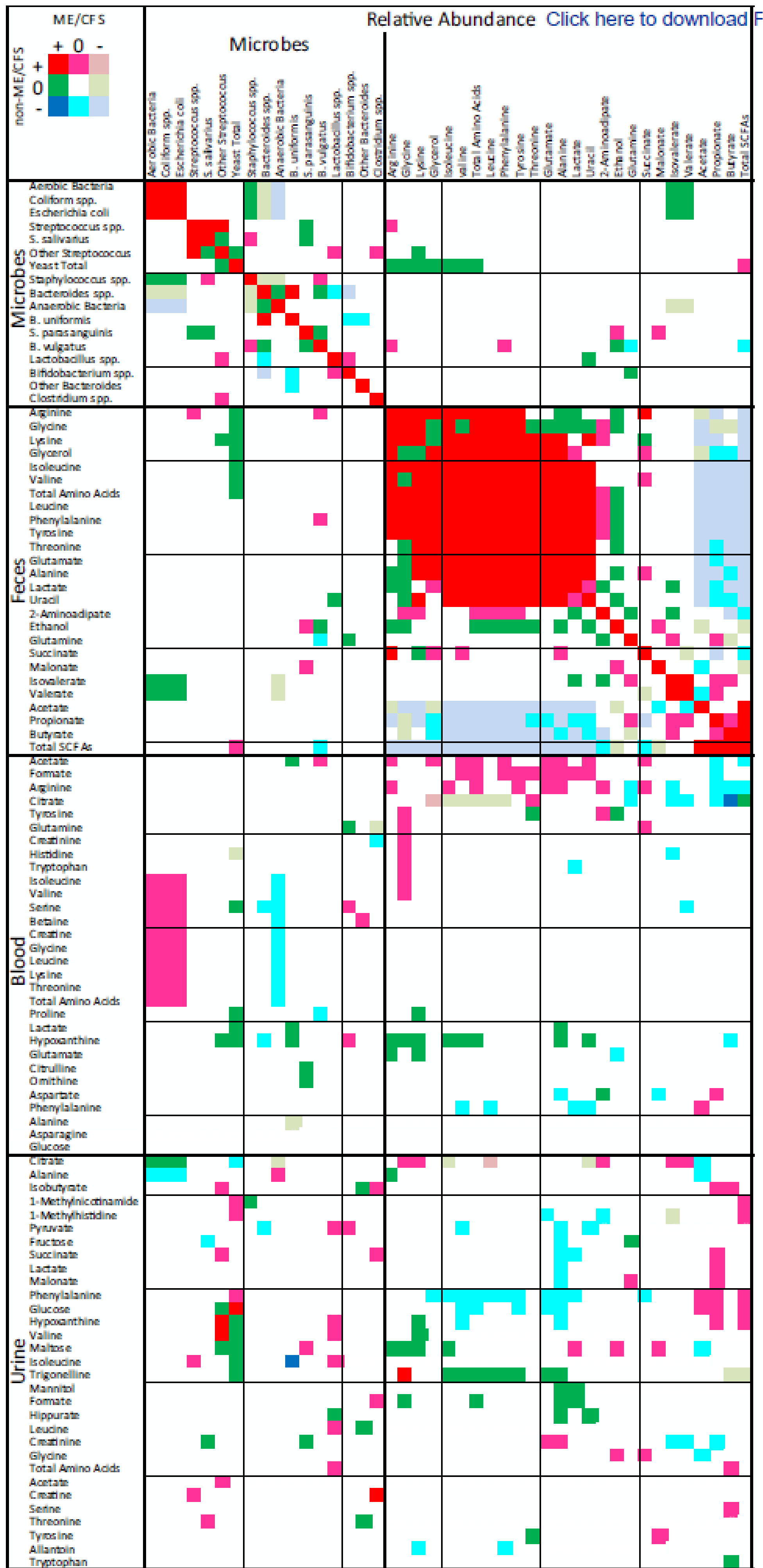
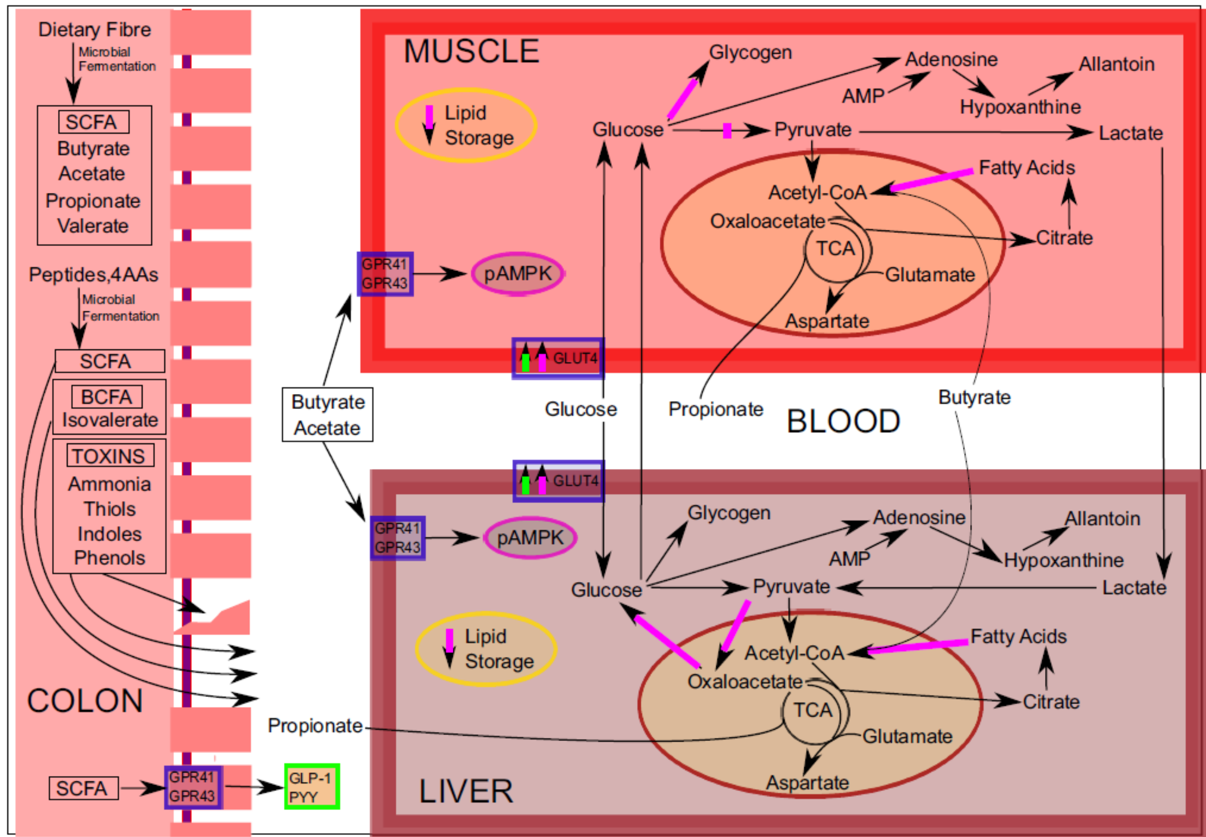

ME/CFS
+ 0 -
non-ME/CFS
+
0
-
Relative Abundance
Click here to download F
Microbes
Aerobic Bacteria
Coliform spp.
Escherichia coli
Streptococcus spp.
S. salivarius
Other Streptococcus
Yeast Total
Staphylococcus spp.
Bacteroides spp.
Anaerobic Bacteria
B. uniformis
S. parasanguinis
B. vulgatus
Lactobacillus spp.
Bifidobacterium spp.
Other Bacteroides
Clostridium spp.
Arginine
Glycine
Lysine
Glycerol
Isoleucine
Valine
Total Amino Acids
Leucine
Phenylalanine
Tyrosine
Threonine
Glutamate
Alanine
Lactate
Uracil
2-Aminoadipate
Ethanol
Glutamine
Succinate
Malonate
Isovalerate
Valerate
Acetate
Propionate
Butyrate
Total SCFAs
Microbes
Aerobic Bacteria
Coliform spp.
Escherichia coli
Streptococcus spp.
S. salivarius
Other Streptococcus
Yeast Total
Staphylococcus spp.
Bacteroides spp.
Anaerobic Bacteria
B. uniformis
S. parasanguinis
B. vulgatus
Lactobacillus spp.
Bifidobacterium spp.
Other Bacteroides
Clostridium spp.
Feces
Arginine
Glycine
Lysine
Glycerol
Isoleucine
Valine
Total Amino Acids
Leucine
Phenylalanine
Tyrosine
Threonine
Glutamate
Alanine
Lactate
Uracil
2-Aminoadipate
Ethanol
Glutamine
Succinate
Malonate
Isovalerate
Valerate
Acetate
Propionate
Butyrate
Total SCFAs
Blood
Acetate
Formate
Arginine
Citrate
Tyrosine
Glutamine
Creatinine
Histidine
Tryptophan
Isoleucine
Valine
Serine
Betaine
Creatine
Glycine
Leucine
Lysine
Threonine
Total Amino Acids
Proline
Lactate
Hypoxanthine
Glutamate
Citrulline
Ornithine
Aspartate
Phenylalanine
Alanine
Asparagine
Glucose
Urine
Citrate
Alanine
Isobutyrate
1-Methylnicotinamide
1-Methylhistidine
Pyruvate
Fructose
Succinate
Lactate
Malonate
Phenylalanine
Glucose
Hypoxanthine
Valine
Maltose
Isoleucine
Trigonelline
Mannitol
Formate
Hippurate
Leucine
Creatinine
Glycine
Total Amino Acids
Acetate
Creatine
Serine
Threonine
Tyrosine
Allantoin
Tryptophan

Figure 4

Click here to download Figure Figure-4-paul.eps



718