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1 **Biology of the Microbiome 2. Metabolic role.**

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11 **Key Points**

12 Microbiome, metabonome, 'omic approaches, metabolic interactions

13 ***Both these authors contributed equally to this manuscript.**

14 **Synopsis:** The human microbiome is a new frontier in biology and one that is helping to define
15 what it is to be human. Recently we have begun to understand that the “communication”
16 between the host and its microbiome is via a metabolic super-highway. By interrogating and
17 understanding the molecules involved we may start to know who the main players are, how
18 we can modulate them and mechanisms of health and disease.

19 **Keywords:** Metabonome, microbiome, mass spectrometry, NMR, multi-variate data.

20

21 **Introduction**

22 Understanding mammalian biology has for the best part of 100 years been focused on trying
23 to model how this system interacts with the environment. Since the discovery that DNA
24 contains all the necessary information to recreate a new living organism and coupled with the
25 revolution in gene sequencing, this focus turned to try to understand how host's genome
26 interacts with its environment to influence the balance between health and disease. A
27 significant component of this body of work has been focused on the role of microbial
28 pathogens in driving disease phenotypes. However, there is a dearth of information which
29 considers that the microbes colonising the various niches of the human body may actually
30 have co-evolved with the host and provide essential functions not found in the host's genome.
31 The role of mammalian microbiome is revealed, *in extremis*, when animals are reared in a
32 sterile environment ^{1,2} and thus do not develop in the presence of a microbiota. The absence
33 of the microbiome has been shown to influence a very wide and disparate range of
34 physiological parameters, including cardiac size and output ³, response to anaesthesia ⁴ and
35 many other features of the mature mammalian system ⁵. While we can see a fundamental
36 role for the microbiome in the development of a mature host we are left with a dearth of
37 mechanisms by which this process is driven.

38 ***Why do we need to know what metabolites are made?***

39 The history of microbiology has been predominantly focused on understanding the role that
40 pathogens play in disease, and this goes back to the time of Robert Koch and Louis Pasteur.
41 However, in the last 15 years there has been a slow, but inexorable move towards
42 understanding how the commensal and mutualistic members of the human microbiome also
43 contribute to host health and disease initiation. In the last five years this interest in the
44 microbiome has really expanded at an exponential rate. However, we cannot treat these
45 organisms in a similar fashion to pathogenic microbes, since they have not evolved specific
46 strategies to invade, colonise and reproduce in a hostile environment. Many of the functions

47 and features that they possess, and on which we rely, do not conform to the virulence model
48 that we have used to describe and understand pathogens. Many of the functions are actually
49 part of that everyday metabolism of these organisms and as such cannot be considered as
50 virulence factors. For example, for many anaerobic bacteria which colonise the large intestine
51 the ability to ferment simple molecules, to extract energy from them, results in a wide range of
52 metabolites which are bioactive and interact with a wide range of receptors within the host.
53 Thus the communication between this diverse set of organisms and its host is predominantly
54 via a metabolite super-highway. Thus in order to understand this communication we need to
55 be able to characterise the wide array of metabolites that the bacteria produce in response to
56 the environment in which they find themselves and understand how the host responds to these
57 metabolites based on the genes that they have.

58 ***How do assess them and what can we assess – NMR and MS, what samples?***

59 Microbial metabolites are typically present in faeces, luminal contents and blood, particularly
60 the hepatic portal vein blood, whilst host-microbial co-metabolites are present more commonly
61 in circulating blood and urine. Metabolic profiling approaches are increasingly used to study
62 metabolic function of the gut microbiota. The practical implementation of metabolic profiling
63 includes five steps: **(1)** sample collection and preparation; **(2)** biochemical composition
64 analyses; **(3)** data analysis and integration (e.g. statistically correlating metabolic and
65 microbial data); **(4)** biomarker recovery and identification; and **(5)** validation and application.

66 Urine and blood plasma or serum collection is straightforward, whereas obtaining faecal
67 samples is more challenging and rarely done at outpatient clinics. Moreover, faecal samples
68 are complex in nature since they contain microbial and mammalian cells and food residues,
69 in which the biological and chemical processes continue during post-voiding and sample
70 handling. Hence, storing samples at a lower temperature and immediate sample processing
71 reduce the variation induced by sample handling. Standard operating procedures for biofluid
72 collection and the effects of various handling conditions on the biochemical composition have

73 previously been reported⁶⁻⁸. Analytical platforms, including nuclear magnetic resonance (NMR)
74 spectroscopy and mass spectrometry (MS), are commonly used in metabolic profiling and can
75 detect a wide range of microbial metabolites and host-microbial co-metabolites. NMR
76 spectroscopy is a robust analytical platform with high reproducibility and it generates the most
77 easily accessible and comprehensive information on metabolite structures. Although the
78 sensitivity of NMR spectroscopy is less than mass spectrometry, it is non-destructive and
79 requires minimum sample preparation. A single proton (¹H) NMR experiment using a 600 MHz
80 NMR spectrometer takes about 5-10 minutes and can detect a wide range of metabolites
81 including amino acids, fatty acids, phenols, indole and other organic acids containing protons
82 at low-micromolar levels. Therefore, it serves as the first choice for global profiling. Mass
83 spectrometry provide complementary molecular information (e.g. molecular mass) and it is
84 much more sensitive than NMR spectroscopy, but often requires pre-separation techniques
85 such as liquid chromatography (LC) and gas chromatography (GC). Depending on the
86 metabolites of the interest, different methods can be employed in liquid chromatography to
87 focus on subsets of molecules. For example, reversed phase chromatography (RP-LC) is used
88 to study non-polar compounds whereas hydrophilic interaction liquid chromatography (HILIC)
89 is used for detecting polar compounds. Both RP-LC-MS and HILIC-MS are routinely used to
90 analyse the same sample sets to achieve wider metabolite coverage. GC-MS is also a
91 sensitive tool in metabolic profiling and commonly used to quantify short chain fatty acids.
92 However, the drawback of GC-MS is that it requires derivatisation of the samples, a long
93 sample preparation procedure, and only volatile compounds or compounds that are volatile
94 after derivatisation can be detected. The main metabolic profiling platforms and their strengths
95 and limitations have been summarised by Holmes *et al.* 2015⁹. Detailed experimental
96 protocols for global metabolic profiling¹⁰⁻¹² and bile acid profiling¹³ have been published.

97 All of these analytical tools generate signal-rich data, which requires multivariate statistical
98 analyses to extract useful information from the datasets. Multivariate data analysis methods,
99 typically including principal component analysis (PCA), orthogonal projections to latent

100 structures-discriminant analysis (O-PLS-DA) and random forest, provide easy visualisation of
101 the metabolic similarities and differences between the samples or spectral data. O-PLS
102 regression analysis is also used to statistically correlate metabolic data with other types of
103 datasets, such as body weight, histological scores, bacterial counts generated from 16S rRNA
104 gene based sequencing platform, cytokines, toxicity, see Fig 1 as an example. Such
105 correlation analysis between metabolic and microbial datasets allows further insight on
106 metabolites that are likely to be associated with gut microbial composition. The statistical
107 modelling results in a panel of spectral signals that are important for class discrimination (*e.g.*
108 treatment group vs. control patients). Signal or feature identification can be challenging in
109 global metabolic profiling. There are many publically available databases such as human
110 metabolome database ¹⁴ and METLIN ¹⁵, software including Chenomx NMR Suite (Chenomx
111 Inc.) and AMIX (Bruker) and published literature sources, which can assist in providing
112 metabolite candidates for the selected features. Statistical total correlation spectroscopy
113 (STOCSY) analysis is a statistical tool to calculate correlation between the peaks from the
114 same molecules or the same biological pathways ^{16,17}. Further analytical experiments should
115 be carried out to confirm the metabolite identification. Various two-dimensional NMR
116 spectroscopic experiments can be used to elucidate the connectivity of protons and carbons
117 of the metabolites. Tandem MS/MS can be employed to obtain fragmentation patterns of the
118 selected MS features to provide sub-molecular information for metabolite identification. In the
119 case of targeted signals or metabolites at very low concentrations, solid phase extraction is
120 often used to separate the signals of interest and concentrate it up for further 2D NMR
121 experiments. In addition, metabolite candidates can be confirmed by spiking the standard
122 compounds in the original biological samples and being tested by NMR spectroscopy or
123 comparing the LC retention times and MS fragmentation patterns from the standards and the
124 samples. Metabolite identification is a time consuming step and is considered to be a bottle
125 neck in the metabolic profiling approach. These metabolite identification methods are often
126 combined in order to elucidate the structure of the targeted spectral signals. Approximate
127 numbers of metabolites seen in different biofluids can be in the range of thousands for both

128 urine¹⁸ and serum¹⁹. Statistical validation can be carried out using methods such as N-fold
129 cross validation and permutation testing, whereas biological validation remains challenging
130 due to further requirement of knowledge of the target metabolic pathways, appropriate
131 validation approaches and additional resources. Statistically and biologically validated output
132 from metabolic profiling may eventually be applied to further mechanistic investigation, and
133 clinical diagnosis and therapeutic decision making.

134 ***Examples of using metabolic profiling to study gut microbial functionality***

135 The advancement of systems biology techniques, in particular metabolic profiling
136 (metabolomics/metabonomics) and mathematical modelling approaches, has expanded the
137 resolution at which we can study the metabolic contribution of the gut microbiota and their
138 interaction with host biochemistry. A key strength of metabolic profiling is its holistic nature,
139 simultaneously capturing vast amounts of metabolic information without bias, surpassing the
140 need for a specific hypothesis allowing open questions to be asked. This property is ideal for
141 studying the gut microbiota due to its mega-variate host-specific nature and our relatively
142 limited understanding. Instead metabolic profiling is a hypothesis generating top-down
143 approach that can illuminate linkages between the gut microbiota and host metabolic
144 pathways for further evaluation.

145 Coupling these data-rich techniques with gnotobiotic (aka germ-free or sterile) and antibiotic-
146 treated animal models has allowed these biochemical associations to be elucidated and their
147 relevance to health and disease to be studied. Pair-wise comparisons of the plasma metabolic
148 phenotypes between gnotobiotic and conventionalized mice using an LC-MS and GC-MS-
149 based approach highlighted the influential role of the gut microbiota on circulating amino acids
150 and organic acids²⁰. Differences were observed in the plasma levels of bioactive indole-
151 containing metabolites derived from tryptophan such as indoxyl sulphate and indole-3-
152 propionic acid. The absence of these metabolites in the gnotobiotic animals, coupled with their
153 greater abundance of tryptophan, indicates that this tryptophan metabolism is dependent upon

154 the gut microbiota. Certain bacteria possess tryptophanase activity (a deamination of the
155 amino acid) and can break down dietary tryptophan to indole. This molecule can be absorbed
156 from the gut and metabolized in the liver to indoxyl before being sulphated to indoxyl-sulphate.
157 Indole can also be further processed by a different set of intestinal bacteria to the antioxidant
158 indole-3-propionic acid. The plasma of gnotobiotic animals also contained greater amounts of
159 the amino acid tyrosine while the conventional plasma contained greater amounts of the
160 microbial-host co-metabolite 4-cresyl-sulphate. Intestinal bacteria have been shown to
161 metabolize dietary tyrosine to 4-cresol, which upon absorption from the gut is sulphated in the
162 liver to 4-cresyl sulphate (*p*-cresyl sulphate). These findings demonstrate the influence of the
163 gut microbiota on the bioavailability of dietary amino acids, precursors for a range of essential
164 bioactive metabolites.

165 Similarly, a ¹H NMR spectroscopy-based metabonomic approach was used to characterize
166 the changes in the urinary metabolic profiles of gnotobiotic rats during 21 days of microbial
167 colonization ²¹. Here, the acquisition of the gut microbiota was accompanied by marked
168 changes in the urinary biochemical profile. Elevations were noted in the excretion of hippurate,
169 phenylacetyl-glycine, and 3- and 4-hydroxyphenylpropionic acid (3-HPPA, 4-HPPA). These are
170 microbial-host co-metabolites that result from the microbial metabolism of dietary components.
171 Phenylacetyl-glycine arises from the bacterial metabolism of the amino acid phenylalanine to
172 phenylacetate, which is conjugated with glycine in the rat liver to form phenylacetyl-glycine and
173 with glutamine in the human liver to form phenylacetyl-glutamine. Hippurate is the glycine
174 conjugate of benzoic acid, which can be derived from the bacterial metabolism of
175 phenylalanine, chlorogenic acid and catechins. These molecules can be obtained from a range
176 of polyphenolic compounds found in dietary components such as fruit, vegetables, tea and
177 coffee ²². Interestingly, in a large-scale metabolic phenotyping study in humans from China,
178 Japan, United Kingdom and the United States, hippurate excretion was found to be inversely
179 associated with blood pressure, a major risk factor for cardiovascular disease ²³. Formate, a
180 product of gut microbial fibre fermentation, was also inversely associated with blood pressure.

181 Another metabonomic study characterized the systemic metabolic adaptation to gut
182 colonization in gnotobiotic mice ²⁴. Following 5 days of conventionalization, the metabolic
183 strategy of the liver shifted from glycogenesis to lipogenesis. This observation was consistent
184 with another study combining a transcriptomic and metabonomic approach to study metabolic
185 response to colonization in the mouse jejunum. Here, two days of colonization resulted in the
186 suppression of lipid catabolism (e.g. β -oxidation) in the jejunum and activation of anabolic
187 pathways (e.g. lipogenesis, nucleotide synthesis and amino acid synthesis) ²⁵. Such
188 biochemical reorientations occurred in parallel to a rapid increase in body weight. These
189 observations indicate the intimate biochemical relationship between the gut microbiota and
190 host and how the host metabolic phenotype is shaped with the development of the gut
191 microbiota.

192 Antibiotic-treated animal models offer another tool for investigating microbial-host interactions.
193 Gnotobiotic animals differ phenotypically from conventional animals raised in the presence of
194 bacteria. Gnotobiotic animals have a reduced body weight, a lower metabolic rate,
195 underdeveloped gut structure and absorptive capacity, and an immature immune system and
196 as such can obscure the interpretation of results. Administering antibiotics to conventionally
197 raised animals allows the influence of the gut microbiota on host biochemistry to be studied
198 whilst preserving the conventional phenotype. This influence was demonstrated by
199 administering the broad-spectrum antibiotics streptomycin and penicillin in the drinking water
200 of rats for eight days ²⁶ and in an early study vancomycin to mice ²⁷. Swann and colleague
201 used ¹H NMR spectroscopy to compare the urinary and faecal metabolic profiles of control,
202 antibiotic suppressed and a group undergoing recolonization (4 days of antibiotics followed by
203 4 days of control treatment). In this study, antibiotic-induced suppression of the intestinal
204 microbiota reduced the urinary excretion of hippurate, phenylpropionic acid,
205 phenylacetyl glycine, indoxyl-sulphate, trimethylamine-*N*-oxide (TMAO) and the short chain
206 fatty acid (SCFA), acetate. The excretion of the amino acids taurine and glycine, and the TCA
207 cycle intermediates, citrate, 2-oxoglutarate, and fumarate was increased following microbial

208 attenuation. In addition, all the SCFA (acetate, butyrate, propionate) were reduced in the
209 faeces of the antibiotic-treated rats. SCFA arise from the bacterial fermentation of
210 carbohydrates, including non-digestible polysaccharides. As these products provide a
211 significant energy source for the host, this represents a key function of the gut microbiota
212 salvaging energy from the diet. A human study by Claesson *et al.*²⁸ correlated faecal metabolic
213 and microbial profiles to highlight a putative statistical association between butyrate and the
214 presence of *Ruminococcus* or *Butyricoccus*. Microbial and metabolic profiling of the
215 recolonizing animals revealed a cage-dependent bacterial recolonization. This difference was
216 mirrored by cage-dependent differences in the metabolic signatures. This highlights the
217 potential for environmental pressures to shape the gut bacterial re-establishment post-
218 antibiotic therapy with downstream implications on the metabolic state of the host.

219 In addition to global profiling of low molecular weight metabolites, we can also target specific
220 molecules or families of molecules, for example, bile acids and eicosanoids. Targeted profiling
221 of the bile acid signature enables a detailed overview of the enterohepatic circulation to be
222 gained and the influence of the gut microbiota to be studied¹³. The circulating and hepatic bile
223 acid pool contains more than 30 known bile acids and the gut microbiota is responsible for
224 driving the majority of this diversity²⁹. Primary bile acids (cholic acid and chenodeoxycholic
225 acid) are synthesized in the liver from cholesterol and are conjugated with either taurine or
226 glycine before secretion into the bile. Upon ingestion of a meal, bile acids stored in the gall
227 bladder are expelled from the gall bladder into the small intestine and although the majority
228 are actively absorbed in the small intestine a minor amount (1-5%; 200-800 mg daily in
229 humans) reaches the colon. It is here that bile acids are modified by the resident microbiota.
230 Many bacteria possess bile salt hydrolase (BSH) enzymes that deconjugate the bile acid from
231 its amino acid. Once deconjugated further bacterial modifications can occur such as
232 dehydroxylation giving rise to secondary bile acids such as deoxycholic acid and lithocholic
233 acid. Modified bile acids can be absorbed and recycled to the liver where they are
234 reconjugated and secreted into the bile. This absorption forms the enterohepatic circulation

235 whereby molecules are shuttled between the host liver and the microbiome. While bile acids
236 have a key role in lipid digestion and absorption they are now also recognized as important
237 signalling molecules serving as ligands for the nuclear receptor; farnesoid X receptor (FXR),
238 and the plasma membrane bound G protein-coupled receptor, TGR5^{30,31}. Through binding to
239 these receptors bile acids can regulate genes involved in lipid³²⁻³⁴ and glucose metabolism
240^{35,36} and energy homeostasis³⁷. Using a parallel transcriptomic and metabonomic approach
241 the influence of the gut microbiota on the enterohepatic circulation and its signalling capacity
242 was studied³⁸. An LC-MS based approach identified pronounced variation in the bile acid
243 signatures of conventional and gnotobiotic rats with similar modulations induced by antibiotic
244 treatment. The absence or attenuation of the gut microbiota shifted the bile acid signature to
245 one dominated by taurine-conjugated bile acids and strikingly reduced the diversity of the bile
246 acid pool. Such modulations impacted on the signalling function of the bile acid profile with
247 significant alterations in the expression of genes and pathways regulated by bile acids. In
248 addition to being measured in the blood and liver, bile acids were also measured in tissues
249 outside of the enterohepatic circulation (kidney, heart) indicating a broader signalling role of
250 these microbial-host co-metabolites.

251 Metabolic profiling strategies applied to human studies have also expanded our understanding
252 of the gut microbial contribution to host digestion and metabolism. This is well illustrated by
253 the microbial metabolism of dietary choline to trimethylamine (TMA). Choline is predominantly
254 derived from phosphatidylcholine found in animal sources in the diet. The microbial
255 metabolism of choline involves the cleavage of the C-N bond to liberate TMA and
256 acetaldehyde. While acetaldehyde undergoes further microbial metabolism to ethanol, TMA
257 is absorbed from the gut and oxidized in the liver to form trimethylamine-*N*-oxide (TMAO) by
258 the flavin-containing monooxygenase 3 (FMO3) enzyme. TMA can also be demethylated to
259 dimethylamine both endogenously and by the gut microbiota (PMID: 4091797). Microbial
260 processing of choline is well established³⁹ and TMA and TMAO have been previously
261 observed in biofluids from gnotobiotic and antibiotic-treated rodents^{21,26,40}. However, recent

262 work in humans has linked this activity to increased cardiovascular disease (CVD) risk. In a
263 global metabolic profiling study in humans, Wang *et al.* ⁴¹ found that three plasma metabolites
264 were predictive of CVD, choline, its metabolite betaine, and TMAO. The role of these
265 metabolites in CVD risk was investigated by feeding them individually to mice. Both choline
266 and TMAO were found to promote atherosclerosis and all three metabolites up-regulated the
267 expression of macrophage scavenger receptors known to contribute to the atherosclerotic
268 process. The essential role of the gut microbiota in potentiating the bioactivity of choline
269 through TMA production was confirmed using gnotobiotic mice. In a metabolic profiling study,
270 microbial choline metabolism has also been shown to exacerbate non-alcoholic fatty liver
271 disease (NAFLD), a condition caused by choline deficiency, in mice ⁴².

272 The potential for the gut microbiota to influence host drug metabolism has been demonstrated
273 in a human study characterising the metabolic fate of paracetamol/acetaminophen ⁴³. The
274 metabolic output of the gut microbiota, specifically 4-cresol, was found to influence the phase
275 II detoxification of this widely used analgesic. 4-cresol has toxic properties and requires
276 detoxification by the host. The primary route of this detoxification is sulfation (both in the
277 gastrointestinal tract and in the liver) before excretion in the urine. This is also the preferred
278 route of detoxification for acetaminophen and both molecules are sulphated by the same
279 human cytosolic sulfotransferase, SULT1A1. As these two molecules compete for binding
280 sites as well as for sulphate, the 4-cresol output of the gut microbiota can influence the ability
281 of the host to sulphate acetaminophen. Alternative routes of detoxification including
282 glucuronidation and phase I metabolism by the cytochrome P450 enzymes. Importantly,
283 phase I metabolism results in the generation of the toxic intermediate, *N*-acetyl-*p*-
284 benzoquinone imine (NAPQI). In this study, individuals excreting high amounts of 4-cresol
285 before receiving a standard dose of acetaminophen were found to excrete lower amounts of
286 acetaminophen sulphate and higher amounts of acetaminophen-glucuronide. Such an
287 observation is not limited to acetaminophen and many xenobiotics are detoxified *via* sulfation.
288 Interestingly, using a molecular epidemiology approach we have also observed 4-cresyl

289 sulphate excretion to be positively correlated with age. This observation was found in both a
290 US and Taiwanese populations suggesting that this age-associated change in the metabolic
291 functionality of the gut microbiome is independent of diet and cultural influences. This data
292 has particular relevance given the greater use of drug therapy with aging ⁴⁴. The influence of
293 the gut microbiota on idiosyncratic drug responses has also been demonstrated in a rodent
294 study with the hepatotoxin, hydrazine ⁴⁰. In this metabolic profiling study the protective effect
295 of an established microbiome was demonstrated in rats with gnotobiotic animals showing a
296 marked toxic response to a typically sub-toxic dose. These studies demonstrate the potential
297 of using a global metabolic profiling approach to characterize the metabolic functionality of the
298 gut microbiota to predict the efficacy and safety of orally administered xenobiotics. This
299 represents a step towards a precision medicine approach tailoring pharmacological
300 interventions to the metabolic status of the complete biological system including contributions
301 from the host genome and the microbiome.

302 ***Future directions***

303 To maximise the potential of metabonomic approaches and using them for defining the role
304 which microbes play in maintaining health and driving disease we predict that the follow areas
305 of research will need to be developed.

- 306 • High throughput profiling of cellular responses to metabolites, currently we do not have
307 platform which allow us to measure the responses of different cell types, e.g. colonocytes
308 or hepatocytes to doses or combinations of metabolites.
- 309 • A metabolic lexicon of bacteria – who makes what and from what substrate. The range of
310 metabolites that different microbes make and from what, so we can predict how changes
311 in the composition of the microbiota affects the metabonome is needed.
- 312 • The interactions between bacteria and their combined impact on the host. Many studies
313 look at single organisms, but we are far from understanding how the microbes interact
314 with each other and how this network affects the host via the metabolite axis.

315

316 **Conclusions**

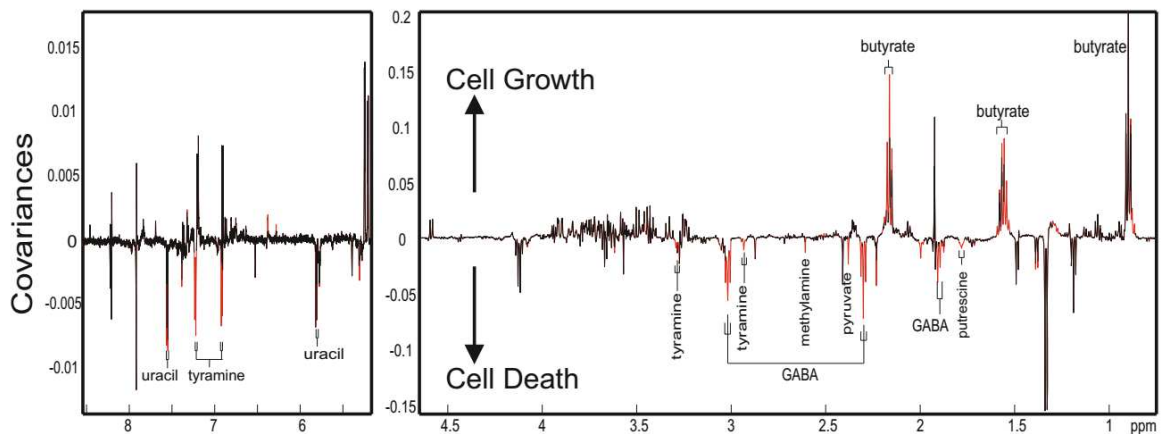
317 To understand how humans function now needs a systems based approach which
318 incorporates the microbiome and its associated metabonome. The metabolic super-highway
319 is the key avenue along which microbes influence the host's metabolism and physiology. In
320 order to understand humans we must start to understand and incorporate this knowledge into
321 our model of the biology otherwise we will still be scrabbling around for explanations for
322 disease for many years to come.

323

324 **Figure Legends**

325 **Figure 1.** O-PLS regression analyses of faecal water from a rat model of bariatric surgery
326 against relative suspension growth values obtained from a 24-h treatment of L5178Y cells.
327 Peaks pointing upward in the loadings plots represent metabolites which are positively
328 correlated to the cell growth and *vice versa*. Red peaks reach a significance level of $p < 0.005$.
329 Keys: GABA, gamma-aminobutyric acid; IS, indoxyl sulfate; PAG, phenylacetylglycine; p-
330 cresyl sulf, p-cresyl sulfate; p-cresyl glu, p-cresyl glucuronide. (Reproduced with permission
331 from ⁴⁵ and modified).

332



333

334

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