

*Dominant components of the
Thoroughbred metabolome characterised
by 1H-NMR spectroscopy: a metabolite
atlas of common biofluids*

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1 **Dominant components of the Thoroughbred metabolome characterised by ¹H-NMR**
2 **spectroscopy: A metabolite atlas of common biofluids.**

3

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12

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14

15 **Keywords:** horse; metabonomics; metabolomics; metabolites; biofluids; nuclear magnetic
16 resonance

17

18

19 **Summary**

20 **Reasons for performing study:** Metabonomics is emerging as a powerful tool for disease
21 screening and investigating mammalian metabolism. This study aims to create a metabolic

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22 framework by producing a preliminary reference guide for the normal equine metabolic
23 milieu.

24 **Objectives:** To metabolically profile plasma, urine and faecal water from healthy racehorses
25 using high resolution $^1\text{H-NMR}$ spectroscopy and to provide a list of dominant metabolites
26 present in each biofluid for the benefit of future research in this area.

27 **Study design:** This study was performed using seven Thoroughbreds in race training at a
28 single time-point. Urine and faecal samples were collected non-invasively and plasma was
29 obtained from samples taken for routine clinical chemistry purposes.

30 **Methods:** Biofluids were analysed using $^1\text{H-NMR}$ spectroscopy. Metabolite assignment was
31 achieved *via* a range of 1D and 2D experiments.

32 **Results:** A total of 102 metabolites were assigned across the three biological matrices. A core
33 metabolome of 14 metabolites was ubiquitous across all biofluids. All biological matrices
34 provided a unique window on different aspects of systematic metabolism. Urine was the most
35 populated metabolite matrix with 65 identified metabolites, 39 of which were unique to this
36 biological compartment. A number of these were related to gut microbial host co-
37 metabolism. Faecal samples were the most metabolically variable between animals; acetate
38 was responsible for the majority (28%) of this variation. Short chain fatty acids were the
39 predominant features identified within this biofluid by $^1\text{H-NMR}$ spectroscopy.

40 **Conclusions:** Metabonomics provides a platform for investigating complex and dynamic
41 interactions between the host and its consortium of gut microbes and has the potential to
42 uncover markers for health and disease in a variety of biofluids. Inherent variation in faecal
43 extracts along with the relative abundance of microbial-mammalian metabolites in urine and
44 invasive nature of plasma sampling, infers that urine is the most appropriate biofluid for the
45 purposes of metabonomic analysis.

46
Accepted Article

47

48 **Introduction**

49 Metabonomics is a powerful systems biology approach that aims to simultaneously measure
50 all the low molecular weight metabolites present in a biofluid or tissue. This approach to
51 global untargeted characterisation of the metabolic phenotype allows the study of
52 multidimensional biochemical responses of complex biological systems to genetic or
53 environmental stimuli [1]. Metabolic profiling captures information from both intrinsic
54 (genetics, protein expression) and environmental inputs (diet, gut microbiota), providing
55 holistic information on the global system. This strategy has proven highly effective for
56 unravelling the complex metabolic interactions between the mammalian host and its resident
57 gut microbiota. Metabonomics is a tool of particular interest to equine researchers given the
58 vast impact of the equine gut microbiome on the bioavailability of food, medication and
59 energy. Metabonomics, along with other 'omic' technologies such as genomics, proteomics
60 and transcriptomics is increasingly showing potential in clinical settings as both a screening
61 tool and a means for mechanistic elucidation of disease pathways [2-4].

62 To date, there are fewer metabonomic studies exploring veterinary concerns than there are
63 human and rodent studies. The majority of mammalian work has concentrated on laboratory
64 animals [5-7] and animal models with high translatability to human health such as the pig [8;
65 9]. Less attention has been given to herbivorous hind-gut fermenters and the majority of
66 equine metabolic work concentrates on drug detection within the racing industry [10; 11].
67 Equine-specific metabonomic studies include the use of the horse as a model for the
68 metabolic response to a dextrose challenge in type-2 diabetes [12] and a laminitic plasma
69 NMR study using an oligofructose overload model [13]. Other equine studies include
70 metabolic analysis of biofluids in response to age [14] and osteochondrosis [15]. Pappalardo

71 *et al.* have explored metabolic variation in association with breed of horse and importantly
72 revealed significant differences that are likely to be attributed to differing growth rates and
73 protein utilisation [16]. These bodies of equine metabonomic work address the enormous
74 potential for exploring normal equine physiology and pathology-based variation. However,
75 there is still a paucity of baseline data on the metabolic phenotype of horses and this study
76 serves as a reference tool for the Thoroughbred racehorse for clinicians wishing to use
77 metabonomic technologies to complement their research in either a diagnostic or mechanistic
78 capacity.

79 Two major analytical platforms are commonly used for metabolic profiling, nuclear magnetic
80 resonance (NMR) spectroscopy and mass spectrometry (MS). These approaches
81 simultaneously capture quantitative information from a range of low molecular weight
82 metabolites across various different sample types. In metabonomic studies, NMR and MS are
83 used both independently and in tandem to achieve an in-depth coverage of the metabolome.
84 In this study we comprehensively characterise the dominant features within the urinary,
85 plasma and faecal metabolomes of Thoroughbred racehorses using ^1H -NMR spectroscopy, as
86 this provides a reliable, reproducible [17] screening tool and is non-destructive of samples.
87 Thoroughbred racehorses represent a highly uniform equine population both genetically and
88 environmentally. Normal clinical chemistry parameters exist for this equine subtype to assist
89 racing industry standards and this study will build upon data already in the literature and in
90 clinical use.

91

92 **Materials and methods**

93

94 Sample collection

95 From a large-scale study of urinary metabonomics of in-training Thoroughbred across a
96 number of yards, 7 animals were selected at random to provide a baseline metabolic reference
97 point for future comparison. Due to ethical constraints of invasive sampling and logistical
98 limitations of simultaneous collection of all 3 biofluids, 7 animals were selected as
99 representative of a wider population of animals. Early morning free-catch urine and faecal
100 samples were collected into sterile plastic containers and snap-frozen in liquid nitrogen
101 within 2 h. Plasma was obtained from excess clinical samples. Plasma samples were
102 collected into heparinised tubes, spun down to obtain the plasma fraction and snap-frozen
103 within 2 h of collection. All samples were then stored at -80°C prior to NMR analysis.
104 Comprehensive metadata for each horse is shown in Supplementary Item 1 and samples were
105 consistently taken before a morning concentrate feed.

106

107 Sample preparation

108 Plasma and urine sample preparation was carried out as described by Beckonert *et al.*
109 2007[18].

110

111 Faecal samples (100 mg) were combined with 1.7 mm Zirconia beads and 1 mL of distilled
112 water and homogenised in a bead-beater for 10 min and centrifuged at 13,000 *g* for 10 min.
113 Water was evaporated from the samples using vacuum concentrator (Speed-Vac) and then
114 reconstituted in 700 μ L phosphate buffer (pH 7.4; 100% D₂O) containing 1 mM sodium 3-
115 trimethylsilyl-1-[2,2,3,3-²H₄]propionate (TSP).

116

117 Combined sampling approach for 2D NMR Experiments

118 2D spectra were obtained from pooled samples for each sample type to ensure comprehensive
119 capture of metabolites. 50 μ L of urine was pooled from each horse before the addition of 500

120 μL of phosphate buffer (and 500 μL of the resulting volume was added to the 5 mm NMR
121 tube). The same process was repeated for the plasma samples with the addition of 500 μL of
122 D_2O saline solution. For the faecal samples, individual samples were processed as previously
123 described and then pooled after reconstitution with 100 μL of phosphate buffer.

124 Acquisition of 1D ^1H -NMR spectra

125 Spectroscopic analysis of all samples was carried out on a 700 MHz Bruker NMR
126 spectrometer^b operating at 300K and equipped with a 5 mm $^1\text{H}(^{13}\text{C}/^{15}\text{N})$ inverse cryoprobe.

127 Urine and faecal samples

128 Standard one-dimensional ^1H -NMR spectra were acquired for all urine and faecal samples.

129 We employed a standard one-dimensional pulse sequence (noesypr1d) that employs the first
130 increment of a NOE sequence to achieve suppression of the water resonance with water peak
131 suppression using a standard pulse sequence [19]. For each sample, 8 dummy transients were
132 followed by 256 transients and collected in 64K data point. Irradiation of the solvent (D_2O)
133 resonance was applied during presaturation delay (2.0 s) for all spectra. The pulse sequence
134 parameters including the 90° pulse, receiver gain and pulse powers were optimised for each
135 sample set run. The spectral width was 20 ppm for all spectra. The free induction decay (FID)
136 was processed with an exponential line broadening of 0.5 Hz prior to Fourier transformation.

137 Plasma samples

138 Water-suppressed Carr-Purcell-Meiboom-Gill (CPMG) spin-echo spectra were acquired for
139 the plasma samples, Here, 8 dummy scans followed by 256 scans were acquired for each
140 sample in 64k data points with a total spin-spin relaxation delay of 1.5 s and a total delay
141 between pulse cycles of 4.85 s.

142 Acquisition of 2D ^1H -NMR spectroscopy was undertaken with an 800 MHz Bruker NMR
143 spectrometer^b operating at 300K and equipped with a triple-resonance probe (TXI). J-

144 resolved spectroscopy (J-res) spectra were acquired from all biofluid composite samples
145 using 64 transients per increment with 160 increments in the second dimension. The F1 (J-
146 coupling) domain spectral width covered 120 Hz. Prior to the double FT and magnitude
147 calculation, the F1 data was zero-filled to 1024 points. The spectra were then tilted by 45° to
148 provide orthogonality of the chemical shift and coupling constant axes and subsequently
149 symmetrised about the F1 axis. ¹H-¹H Correlation Spectroscopy (COSY) was performed on
150 all 3 types of pooled biofluid samples in order to detect correlations between protons on
151 adjacent carbons. Transients were acquired with 4096 data points (sweep width of 7200 Hz in
152 both axis) with 64 scans per increment and 320 increments in the F1 axis. The relaxation
153 between successive pulse cycles was 2.3 s and were weighted using a sine bell function in T₁
154 and T₂ prior to fourier transformation and subsequently symmetrised about the diagonal axis.
155 Two-dimensional echo/anti-echo ¹H-¹³C heteronuclear single quantum correlation (HSQC)
156 spectra were also obtained. 256 scans were collected (16 dummy scans) at a spectral
157 resolution of 4k in F2 across a spectral width of 12 ppm for ¹H and 170 ppm for the ¹³C axes.
158 An acquisition time of 0.852 s and a relaxation delay of 1.2 s were used and delays were set
159 for a 145 Hz one bond ¹H-¹³C coupling constant. Spectra were zero-filled in the F2
160 dimension by a factor of 2 to 8k, and zero-filling and linear prediction was applied in F1 to
161 result in a resolution of 1k.

162 NMR spectral data pre-processing

163 Data [-1.0 to 10.0 ppm] were imported into MatLab environment (7.0 The Mathworks[©]),
164 where they were automatically phased, baseline-corrected and referenced to TSP (δ 0.00) or
165 glucose (δ 5.233) for plasma using scripts written in-house [20]. To reduce analytical
166 variation between samples the residual water signal (4.67 – 4.98 ppm) was truncated from the

167 data set. Probabilistic-quotient normalisation was used on each biofluid class separately to
168 account for differing sample dilutions [21].

169 Data analysis of biological matrices

170 Unsupervised multivariate analysis was undertaken to visualise clustering and differences
171 between samples [22]. Principal component analysis (PCA) was constructed using unit-
172 variance scaled data (UV) [23].

173 Metabolite assignment of endogenous metabolites was made by compiling the following
174 information from each peak: the chemical shift and relative integral height from 1D spectra,
175 the multiplicity using J-res spectra, the proton coupling information from COSY spectra and
176 carbon shifts from HSQC spectra. This information was then used to search for matching
177 metabolites from in-house databases, online databases (<http://www.hmdb.ca/>) and reference
178 to published literature data [24-26]. Statistical Total Correlation Spectroscopy (STOCSY)
179 was also employed to aid metabolite identification [27]. This method uses statistical
180 connectivity between data points within a spectral profile. However, unlike 2D-NMR this
181 method will also pick up metabolites involved in the same pathways due to biological
182 covariance [28].

183

184 **Results**

185 Assignment of dominant metabolites across easily obtainable biofluids yields a preliminary
186 equine metabolic atlas

187 The majority of known NMR-detectable metabolites were assigned in each biological matrix
188 (Fig 1), with numerical reference to Table 1. Metabolite assignments were performed using
189 two-dimensional correlation (COSY) and J-resolved (J-res) spectroscopy to ascertain peak

190 multiplicity, coupling constants and to overcome peak overlap. This is demonstrated in the
191 COSY (Fig 2A) and the J-res (Fig 2B). Heteronuclear Single Quantum Coherence (HSQC)
192 was also performed to provide ^{13}C shifts and confirm assignments. Putative metabolite
193 assignment was made using ^1H and ^{13}C chemical shifts, peak multiplicity, coupling constants
194 and relative peak integrals. Overall, 102 metabolites were identified by ^1H -NMR in the 3
195 biofluids (Table 1). Detailed assignment information is shown in Supplementary Item 2.
196
197 Cross-compartmental analysis revealed a core metabonome, along with compartment specific
198 metabolites
199 Metabolites assigned to the 3 biofluids were compared to ascertain ubiquitous metabolites
200 and those that were specific to each biological compartment. The metabolic variation across
201 the biological matrices is displayed in a Venn diagram (Fig 3) to easily visualise inter-
202 compartmental overlap. A total of 14 metabolites were ubiquitous to all biofluids, which we
203 will refer to as ‘core’ metabolites. These include energy-related metabolites such as glucose
204 and lactate as well as a number of amino-acids including alanine, arginine, glycine,
205 glutamine, taurine, threonine and valine. The microbial related metabolites acetate, formate
206 and *p*-hydroxyphenylacetate were also conserved across all biofluids studied.
207 PCA revealed that inter-animal metabolic variation was lower than the variation between the
208 different biofluids as visualised *via* clustering in the PCA scores plot (Fig 4A). As would be
209 expected, the PCA samples cluster based on biofluid type. However, importantly the degree
210 of clustering is different amongst biological matrices. Faecal samples demonstrate that they
211 are inherently variable compared to either matched urine or plasma samples. The faecal
212 metabonome displayed relatively higher concentrations of SCFA (butyrate, acetate and
213 propionate) compared to plasma and urine. Urine samples had relatively higher level of gut-

214 microbial co-metabolites such as hippurate, phenylacetylglucine (PAG), *p*-cresyl sulfate and
215 trimethylamine-*N*-oxide (TMAO). In contrast, energy-related metabolites such as glucose and
216 pyruvate were observed in relatively higher concentrations in plasma compared to urine.
217 Variance plots shown in Fig 4A and B display the mean spectra of faeces and urine
218 respectively coloured by the variance, represented as a percentage of the total variance. Here,
219 creatinine can be seen to represent over 10% of the total variance seen within the urinary
220 profiles (Fig 4B) and acetate accounted for over 28% of the total variance observed in the
221 faecal profile (Fig 4A) compared to 1.8% in the plasma profile (not shown).

222

223 *Plasma*

224 Equine plasma contained relatively few metabolites compared to urine and faeces when
225 analysed by NMR spectroscopy, partly as a result of the overlap of signals from
226 macromolecular components such as lipoproteins and low molecular weight chemicals. A
227 total of 38 metabolites were identified (Table 1), and of these 12 were unique to this biofluid,
228 including the amino-acids tyrosine and phenylalanine and ketone bodies (α - and β -
229 hydroxybutyrate). Plasma was observed to contain the highest levels of glucose compared to
230 other biofluids within the data matrix.

231 *Urine*

232 The equine urinary metabolic profile was the most metabolically abundant of all biofluids
233 measured (Table 1). In total 65 metabolites were identified. Of these, 11 were unique to urine
234 and plasma, 3 were present in both urine and faeces and 39 metabolites were specific to
235 urine. Urinary-specific metabolites included a number of aromatic compounds that arise from
236 microbial-host co-metabolism. Hippurate, PAG, *p*-cresyl glucuronide and sulphate were

237 notably prominent in the aromatic region of the horse urine spectra (Fig 1B). Other urine
238 specific metabolites of note include dietary compounds such as proline betaine.

239 *Faeces*

240 The faecal metabotype contained 43 metabolites, including 21 specific to faeces (Fig 3).

241 These metabolites include microbial fermentation products, SCFA (butyrate and propionate),

242 and a number of dietary metabolites (caprylate and arabinose, maltose, glycerol and xylose).

243 Acetate was present in significantly greater concentrations than other metabolites.

244

245 **Discussion**

246 This is the first systematic description of the dominant metabolites of the healthy

247 Thoroughbred racehorse and is important in establishing a metabolic reference from which to

248 compare pathology-related variation. Characterisation of the equine metabolome highlights

249 the diversity of information available in different biological matrices and as such provides a

250 useful guide for researchers. Importantly, as has been shown in other species, urine provides

251 a biological window into host-microbial metabolic interactions in the horse [8]. Although

252 faeces may be considered a more direct representation of microbial metabolism, Fig 4A and

253 B highlights the relatively greater variation in the faecal metabolic profiles compared to

254 plasma and urine. Additionally, microbial-derived compounds absorbed from the gut are

255 commonly not well represented in the faecal signature rendering the biological usefulness of

256 this biofluid in gastrointestinal disease and mammalian-microbial co-metabolism

257 questionable.

258 A ubiquitous metabolome is present amongst mammals. The core equine metabolome

259 comprises metabolites present in all 3 biofluids studied. 14 metabolites were identified

260 including amino-acids, gut microbial metabolites and energy metabolites. Many of these

261 metabolites are ubiquitous, with varied functions and located in numerous tissue types.
262 Similar findings were reported by Merrifield *et al.* with 22 metabolites shared across 4
263 biological matrices in pigs [8].
264 Marked ¹H-NMR metabolic similarities between horses and humans are suggested by
265 observations of a number of shared metabolites. In total, there were 32 plasma, 23 urine and
266 27 faecal NMR detectable compounds common to both horses and humans [29-31].
267 Metabolic consistency was strongest across the plasma profiles. This validates the concept of
268 conservation of mammalian physiology across species and that homeostatic metabolic control
269 is tight in both systems. A lower percentage of the equine faecal and urine metabolic profiles
270 were shared with humans but this was to be expected due to different digestive systems and
271 metabolic pathways. In contrast, ruminants share a number of gut microbial co-metabolites
272 such as hippurate and PAG [32-34]. These herbivores have similar digestive strategies to
273 obtain nutrients from a cellulose-rich diet.
274 Excretory biofluids (urine and faeces) are the most metabolite-rich. The environmentally-
275 determined nature of these biofluids renders them under less tight homeostatic control than
276 that of the plasma. However, urine was also remarkably tightly controlled, given the
277 relatively homogeneous genetic and environmental backgrounds of Thoroughbred racehorses.
278 Differing creatinine excretion in one animal (due to the sample being collected after exercise)
279 was responsible for the increased inter-animal variation observed (Fig 4A). In urine, 39
280 metabolites were identified as being unique whereas 21 metabolites were faeces-specific and
281 12 metabolites were plasma-specific. However, there were a small number of low-
282 concentration unassigned metabolites that have not been included in these counts. All 3
283 biofluids are likely to contain thousands of metabolites which are too dilute to produce a
284 significant NMR signal or were not detected by NMR in this study, but we feel we were able

285 to capture a good representation of the normal equine metabonome and this has been verified
286 by our ongoing work on a larger cohort of animals (data not shown).

287 Inter-compartmental variation is greater than inter-animal variation and is consistent with
288 other mammalian studies [8; 35]. Faecal samples varied from other biological matrices due to
289 the presence of a number of SCFAs, formate and isovalerate. SCFAs are the product of gut-
290 microbial fermentation of dietary fibre and contribute up to 70% of a horse's energy
291 requirements [36; 37]. Acetate level variability is likely to be due to differing bacterial
292 communities and consequently SCFA production. Collection time in relation to feeding time
293 is known to exert an effect on SCFA levels in equine faeces [38]. Samples were consistently
294 taken before a morning feed. However, this variability could be due to the difference in
295 individual intestinal transit times [39; 40]. Other SCFAs were not seen to vary to the same
296 extent as acetate. Butyrate is the main energy source for colonocytes and hence is likely to
297 have been utilised rather than excreted [41]. Butyrate and propionate are extensively
298 metabolised by first-pass metabolism and therefore absent in NMR detectable quantities in
299 urine and plasma samples. Creatinine was the greatest source of variation amongst urine
300 samples. Creatinine is a waste product of muscle metabolism formed from creatine in order to
301 maintain ATP levels during exercise [42]. The concentration of urine and thus metabolites
302 can change dramatically. Normalisation of the data prior to analysis helps to minimise
303 spectral anomalies caused by differences in urinary dilution. Creatinine levels can vary
304 according to factors such as muscle mass, physical exercise, diet, age and muscle damage
305 from previous strenuous exercise [43].

306 The urine metabolome provides a metabolic window into gut microbial co-metabolism.
307 Urine was found to differ from the other biological matrices due to the presence of a number
308 of gut-microbial co-metabolites (hippurate, PAG and *p*-cresyl sulphate and TMAO). These

309 compounds originate from exogenous sources (microbial and dietary) and are incorporated
310 into the host circulation after absorption. They subsequently undergo enzymatic conjugation
311 in the liver and gut mucosa to increase their polarity and enable renal excretion. In mammals,
312 enterohepatic recycling means further metabolism can occur at the gut-level [44]. Hippurate
313 is an aromatic compound predominantly formed from glycine conjugation of dietary or
314 microbial benzoate and PAG is a glycine-conjugated microbial metabolite of phenylalanine
315 metabolism (glutamine in higher apes and humans) [45]. *P*-cresol is formed from bacterial
316 degradation of tyrosine and is subsequently sulfated or glucuronidated in the liver or gut [46].
317 Both forms of conversion occur in rodents and horses, whereas humans predominately form
318 sulphate conjugates and pigs predominately glucuronidate the cresol molecule.
319 Trimethylamine (TMA) is a microbial degradation product of dietary choline; this metabolite
320 is absorbed from the gut and subsequently oxidised in the liver to produce TMAO [47]. A
321 number of anaerobic bacterial populations are known to produce these metabolites including
322 clostridia [48-50]. This taxonomic classification of bacteria has been associated with
323 intestinal disease in horses as well as being part of the normal microflora [51-54].
324 ¹H NMR spectroscopy is a robust method for assessing the inter-animal variation in
325 Thoroughbred racehorses. It is important to address potential sources of variation when
326 assessing metabonomic studies. The sensitive nature of such investigations necessitates the
327 collection of metadata to help explain possible variation between the samples. This
328 information should include details from sample collection, sample storage and run order, as
329 well as information relating to sample subject (health status, age, sex for example). The effect
330 of these is widely reported in the literature [35; 55; 56]. Although the 7 horses were taken
331 from 2 different yards, the samples clustered tightly, highlighting the metabolic uniformity of
332 these Thoroughbred racehorses despite differing age ranges, location and stages of fitness

333 (see Supplementary Item 1). This study was intended to create a reference tool for research
334 into Thoroughbred racehorse metabolism and microbial co-metabolism. Since the samples
335 from this first pilot clustered closely for all 3 biofluids indicating that the dominant
336 metabolites visible by NMR were conserved across animals, it was deemed unnecessary to
337 collect samples from further animals to minimise unnecessary sampling. Although, the
338 sample size in this study is small and only one metabolic snap shot was taken for each horse,
339 the fact that inter-animal variation in metabolic profiles was low, suggests that ‘healthy’
340 Thoroughbreds share a similar metabolic phenotype, which we explored using a range of
341 NMR-based structural elucidation tools including 2-D pulse sequences to elicit carbon-proton
342 correlations and statistical spectroscopy methods. Our future work includes a larger study
343 investigating normal variation amongst different racehorse populations over time.

344 This work comprehensively assigns dominant features of the ^1H NMR spectra of the equine
345 metabonome from plasma, urine and faeces and for the first time provides baseline
346 information for future studies in equine health and disease. Urine and faecal profiles provide
347 an insight into host-microbial metabolic interactions, whereas plasma profiles are more likely
348 to represent host physiological processes. The purpose of this study is to provide an analytical
349 template to researchers thinking about adding metabonomic analysis to their experiments and
350 to indicate which biofluids may be of use to them. We have showed that faecal samples are
351 more variable than either urine or plasma. Plasma is under tight homeostatic control and thus
352 might be expected to show relatively less variation. However, urine, other than one identified
353 outlier, exhibited less variation than the other biofluids under investigation and contributed
354 the greatest number of identifiable metabolites. Further studies are warranted using
355 metabonomic and metagenomic technology to explore the role of gut microbes on equine
356 physiology and metabolism.

357

358

359 **Authors' declaration of interests**

360 No competing interests have been declared.

361

362 **Source of funding**

363 Horserace Betting Levy Board Research Scholarship at Imperial College London.

364

365 **Ethical Animal Research**

366 Sampling and metadata collection were carried out under University of Liverpool ethics

367 approval RETH000363, with the informed consent of the trainer.

368

369 **Acknowledgements**

370 We are grateful to the racehorse trainers and owners who allowed us to sample their horses,

371 to Elizabeth Metcalfe from the Animal Health Trust, Newmarket for technical assistance and

372 Aisling Glennie at Boehringer Ingelheim for help editing the manuscript.

373

374

375 **Authorship**

376 E. Escalona contributed to all sections. J. Leng contributed to study execution, and data

377 analysis and interpretation. J. Swann and A. Dona contributed to study design, study

378 execution, and data analysis and interpretation. C. Merrifield contributed to the preparation of

379 the manuscript. All authors gave their final approval of the manuscript.

380

381

382

383

384 **Figure legends:**

385 **Fig 1:** (A) 700 MHz 1D ^1H -NMR spectrum of urine, (B) CPMG ^1H -NMR spectrum of
386 plasma, (C) 1D ^1H -NMR spectrum of faecal water. All spectra partially labelled according to
387 the assignments made in Table 1 and Supplementary Item 2.

388

389 **Fig 2:** (A) 800 MHz ^1H COSY NMR spectrum of the aromatic region of urine (B) J-res NMR
390 spectrum of plasma highlighting the aliphatic region between 3-4.5 ppm. Key to metabolite
391 identification is provided in Table 1 and Supplementary Item 2.

392

393 **Fig 3:** (A) Venn diagram highlighting the degree of metabolite overlap between biological
394 compartments. The central section represents the number of core metabolites visible across
395 all biological matrices (14 ubiquitous metabolites). The outer circle with numbered slices
396 represents biological matrix specific metabolites that are ordered according to
397 origins/function, and numbered according to metabolites in Table 1. Compartments are
398 colour coded - plasma (red), urine (yellow) and faeces (green). Coloured dots represent
399 metabolic functions, protein and amino-acid metabolism (purple), energy metabolism
400 (orange) and fat metabolism (blue). Asterisk denotes metabolite can be mammalian in origin
401 and a 'd' denotes metabolite can be from dietary origin.

402

403 **Fig 4:** (A) PCA scores plot demonstrating increased faecal variability relative to other
404 biological matrices. Plasma (red), urine (yellow) and faecal (green) samples. Principal
405 component 1 (PC1) accounts for 51%, PC2 for 15%, and PC3 for 5% of the total variation.
406 Variance colour plots indicating the percentage of the total variance for each metabolite in
407 (B) faeces and (C) urine (Mean spectrum is plotted, coloured by variance expressed as a
408 percentage of the total variance).

409

410 **Table 1:** Metabolites identified using ^1H -NMR of equine plasma, urine and faeces.
411 Metabolite numbers correspond with annotated ^1H -NMR spectra in Figs 1, 2 and 3.
412 Metabolites are assigned to biofluids denoted by coloured dots; urine (yellow), plasma (red),
413 faeces (green). The 14 core metabolites are highlighted in purple.

414

415

416 **Manufacturers' addresses**

417 ^aBertin Technologies, Montigny-le-Bretonneux, France

418 ^bBruker, Massachusetts, USA

419 ^cMATLAB, Mathwork, Massachusetts, USA

420

421 Additional Supplementary Items may be found in the online version of this article at the
422 publisher's website:

423

424 **Supplementary Item 1:** The table highlights sample metadata and possible sources of
425 variation within the data set. Yard, gender, age and training schedule are included. Gender is
426 denoted as G=gelding, C=colt.

427

428 **Supplementary Item 2:** Table showing metabolites found in plasma (red circle), urine
429 (yellow circle) and faeces (green circle) using ^1H -NMR spectroscopy. Peak multiplicities and
430 chemical shifts are shown and structural information is also provided. † indicates tentative
431 assignment. Details on each metabolite's origin and function are highlighted as well as a link
432 to the metabolite's page in the hmdb database.

433

434 **Supplementary Item 3:** Table denoting feeding regimes on the 2 yards sampled. Top section
435 highlights concentrate feeds and bottom highlights roughage types offered.

436

437 **Supplementary Item 4:** 800 MHz ^1H COSY NMR spectrum of the aliphatic region of
438 faeces, between 3-4 ppm. Key to metabolite identification is provided in Table 1 and
439 Supplementary Item 2.

440

441 **Supplementary Item 5:** 800 MHz ^1H ^{13}C HSCQ NMR spectrum of the aromatic region of
442 urine, between 8-7 ppm. Key to metabolite identification is provided in Table 1 and

443 Supplementary Item 2.

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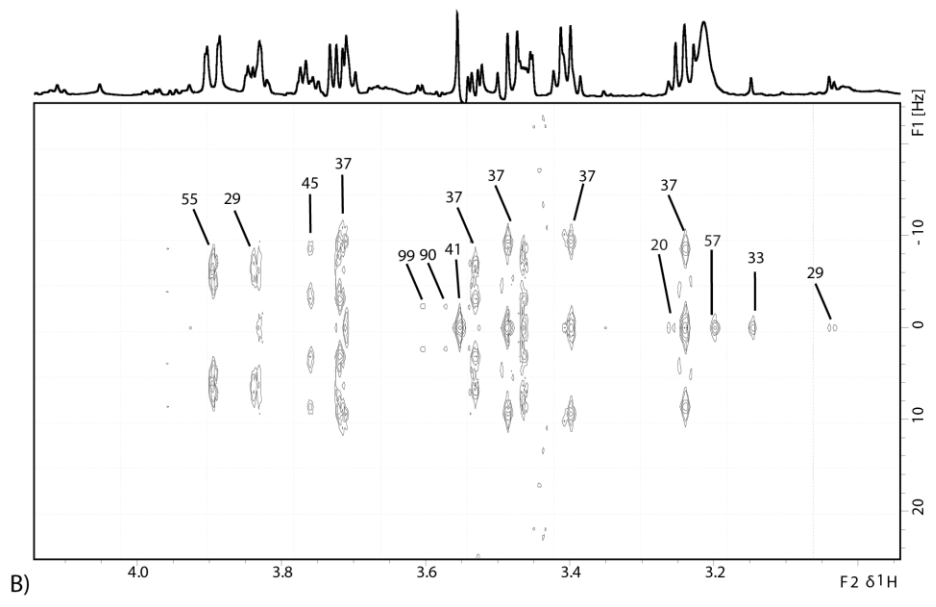
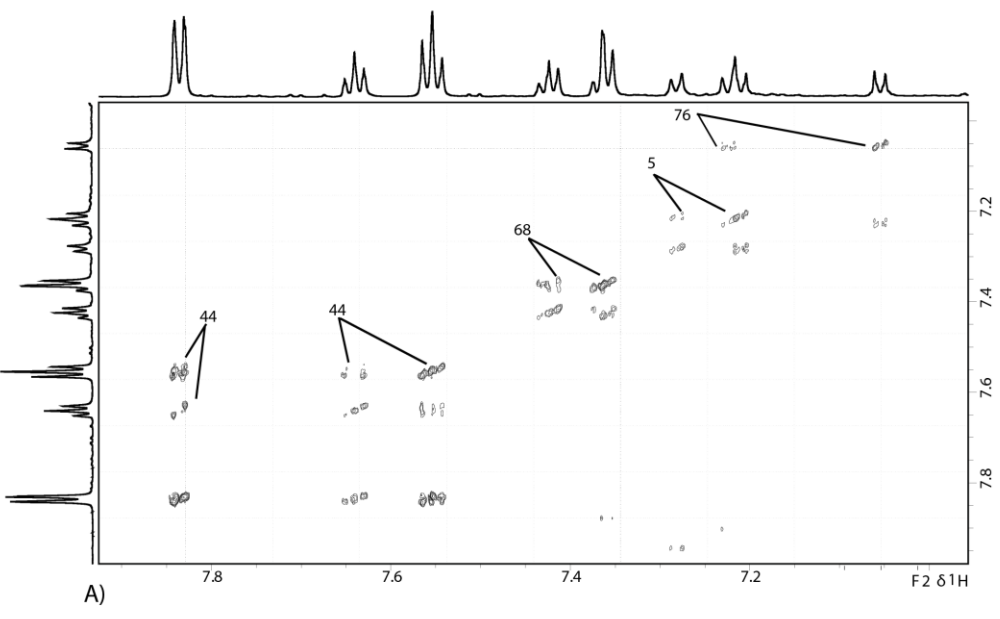
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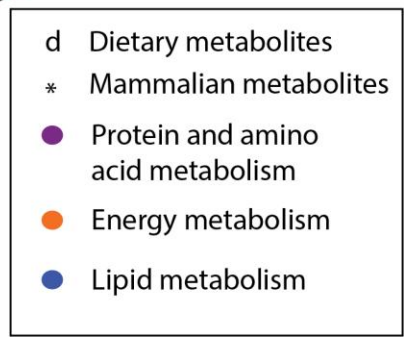
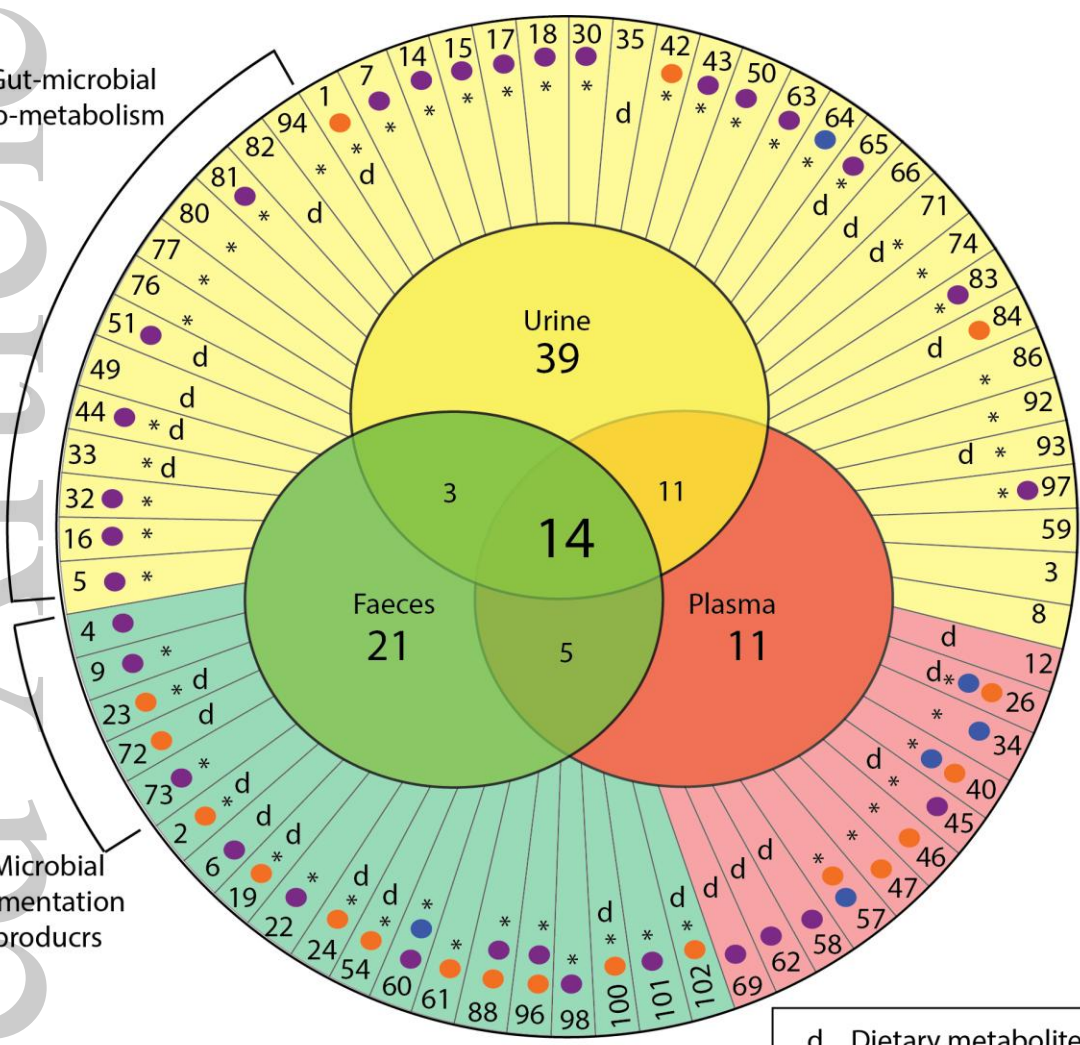


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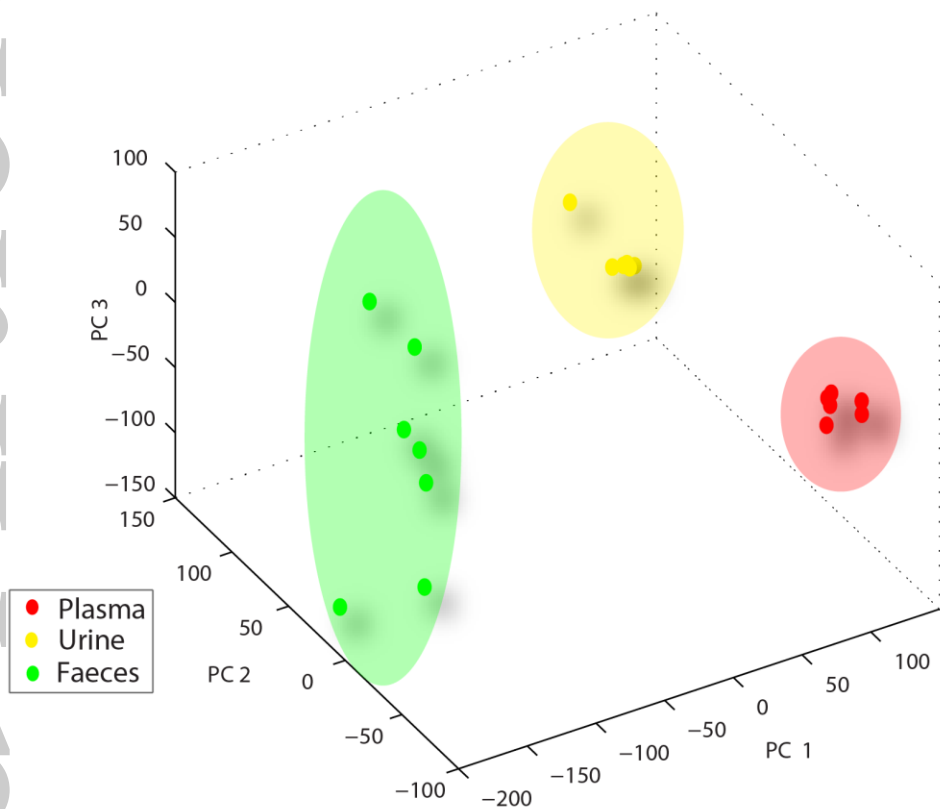
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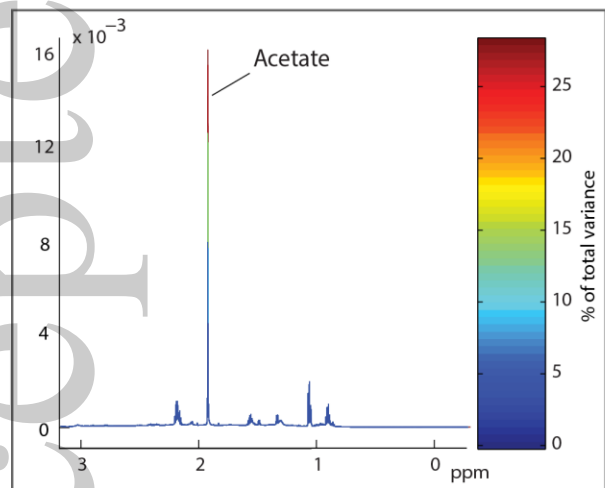


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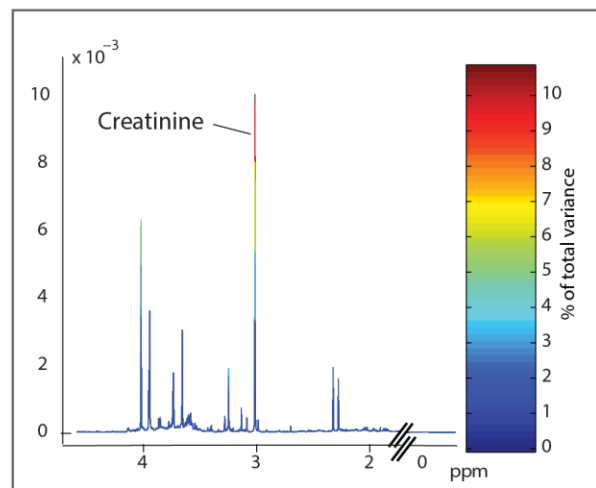
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A)



B)



C)

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N°	Compound
1	2-Hydroxy-3-methylbutyric acid
2	2-Methylbutyrate
3	3-hydroxy-4-methoxymandelic acid
4	3-Hydroxyphenylacetate
5	3-Indoxyl sulphate
6	3-phenylpropionoate
7	3-Ureidopropionic acid
8	4-hydroxy-3-methoxymandelic acid
9	5-Aminovalerate
10	Acetate
11	Acetoacetate
12	Adipate
13	Alanine
14	β Alanine
15	Allantoate
16	Allantoin
17	α -Amino adipate
18	Beta-Aminoisobutyrate
19	Arabinose
20	Arginine
21	Asparagine
22	Aspartate
23	Butyrate
24	Caprylate
25	Choline
26	Cholestrol
27	Citrate
28	Creatine
29	Creatinine
30	Dihydrothymine
31	Dimethylamine (DMA)
32	Dimethylglycine (DMG)
33	Dimethyl sulphone
34	Ethanolamine
35	Ethyl glucoside
36	Formate
37	Glucose
38	Glutamate
39	Glutamine
40	Glycerol
41	Glycine
42	Glycogen
43	Guanidoacetate
44	Hippurate
45	Histidines
46	α Hydroxybutyrate
47	β Hydroxybutyrate
48	Alpha- Hydroxyisobutyrate
49	Hydroquinone
50	Hypotaurine
51	Indole-3-acetate

N°	Compound
52	Isobutyrate
53	Isoleucine
54	Isovalerate
55	Lactate
56	Leucine
57	Lipids
58	Lysine
59	Maleic anhydride
60	Malonate
61	Maltose
62	Methionine
63	Methylguanidine
64	Nicotinurate
65	Orotate
66	Pantothenate
67	Phenylacetate
68	Phenylacetyl glycine (PAG)
67	Phenylalanine
69	Proline betaine
70	Phosphocholine
71	Proline betaine
72	Propionate
73	Putrescine
74	Pyridoxine
75	Pyruvate
76	<i>p</i> -cresol glucuronide
77	<i>p</i> -cresol sulphate
78	<i>p</i> -hydroxybenzoate
79	<i>p</i> -hydroxyphenylacetate
80	<i>p</i> -hydroxyphenyllactate
81	<i>p</i> -hydroxyphenylpyruvate
82	Quinate
83	Quinone
84	Raffinose
85	Scyllo-inositol
86	Sebacate
87	Serine
88	Succinate
89	Taurine
90	Threonine
91	Trimethylamine-N-oxide (TMAO)
92	Trans-aconitate
93	Trigonelline
94	Trimethylamine (TMA)
95	Tyrosine
96	Uracil
97	Urea
98	Uridine
99	Valine
100	Valerate
101	Xanthine
102	Xylose

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