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Characterisation of the faecal metabolome and microbiome of Thoroughbred racehorses

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Summary

Reasons for performing study: The intestinal bacterial community of the horse is a key determinant of intestinal and whole body health. Understanding the bacterial community structure and function is an important foundation for studies of intestinal health and disease.
Objectives: To describe the faecal bacterial community and volatile organic compounds (VOCs) of the faecal metabolome of healthy Thoroughbred racehorses and to characterise responses to dietary supplementation with amylase-rich malt extract.
Study design: Intervention study.
Methods: Faecal samples were collected noninvasively before and 6 weeks after supplementation in 8 privately owned Thoroughbred racehorses in active race training. Faecal metabolome was characterised using thermal desorption-gas chromatography-mass spectrometry (TD-GC-MS), with spectral analysis performed using AMDIS and compared against the NIST database. Taxonomic description of the faecal microbiota was achieved using error-corrected 454 pyrosequencing data from 16S rRNA gene amplicons.
Results: The faecal metabolome of our study population was dominated by organic acids, alcohols and ketones. We identified 81 different VOCs only 28 of which were present in >50% of samples indicating functional diversity. Faecal VOC profiles differed between first and second sampling point, some VOCs being significantly reduced post supplementation, consistent with a marked response to dietary amylase-rich malt extract. Faecal microbiota was characterised as highly diverse; samples demonstrated verifiable diversity in the range 1200–3000 operational taxonomic units (OTUs) per individual. The methods used also describe high levels of infrequent, low abundance OTUs. Faecal microbial community structure was found to be different following dietary supplementation. Differences in several low abundance bacterial taxa were detected and also some evidence of interhorse variation in response.
Conclusions: The volatile faecal metabolome of Thoroughbred racehorses is dominated by organic acids, alcohols and ketones; this study demonstrates that dietary supplementation with amylase-rich malt extract may significantly alter the profile of VOCs. The faecal microbiome is highly diverse, dominated by Firmicutes and Bacteroidetes. Small but significant changes in microbial community structure were detected following dietary supplementation. This study describes the faecal metabolome and microbiome of healthy Thoroughbred racehorses against which future studies of disease and dietary intervention can be benchmarked.

Keywords: horse; faecal; metabolome; microbiome; microbiota; volatile organic compounds

Introduction

There is a growing understanding of the complex interactions between intestinal microbiota (definition: microbial organisms residing in the intestine) and host metabolism [1,2]. Metabolites are highly conserved across species and report directly on the metabolic and physiological status of the subject in health and disease [3–5]. The faecal metabolome (definition: complete set of small molecules metabolites found in a sample) reports specifically on the metabolic interplay between host, diet and intestinal microbiota [6,7]. Modern analytical methods allow the characterisation and quantification of the complete spectrum of metabolites using different analytical platforms, e.g. mass spectrometry, nuclear magnetic resonance spectroscopy [8] and the profiling of complex bacterial communities using next generation sequencing [9].

The faecal volatile metabolome of the horse has not been described and taxonomic characterisation of the faecal microbiome (definition: the collective genomes of the microorganisms that reside here) is limited by small numbers of horses studied and by the methods used. Daly *et al*. [10] described the use of molecular methods to characterise the colonic microbiome of horses *post mortem* and dysbiosis associated with a laminitis model has been studied by denaturing gradient gel electrophoresis (DGGE) and RT-PCR [11]. Community profiling of horse and pony faecal microbiota has been described [12,13] using terminal restriction fragment length polymorphism (T-RFLP) analysis demonstrating differences in community profile in different regions of the hindgut, and temporal stability of individual animal bacterial communities. Willing *et al*. [14] characterised changes in faecal microbiota in Standardbreds in training by T-RFLP and cloning.

The first report of pyrosequencing to characterise faecal bacterial communities in 2 horses was published recently [15] and the method has since been used to identify differences in microbial communities between normal horses and those with colitis [16]. Deep amplicon sequencing using high throughput platforms such as Roche 454, Ion Torrent and Illumina provide a digital count and taxonomic description of microbial communities. Deep sequencing of taxonomically informative genes (i.e. 16S rDNA) has advantages over molecular 'fingerprinting' techniques (e.g. DGGE and T-RFLP) as it characterises the taxa in the community and gives a measure of taxon abundance.

It is widely recognised that diet and change in diet are risk factors for intestinal disease ('colic'), a significant cause of mortality and economic loss in managed horse populations [17–19]. There is good evidence to support the hypothesis that carbohydrate overflow into the caecum and colon is an important mechanism in some types of colic, leading to changes in pH, microbial populations and fermentation products [20–22]. Understanding the structure and metabolic function of normal gut microbiota and how this responds to dietary change is an important first step towards evidence-based dietary intervention to improve health and decrease the risk of disease.

The aim of this study was to characterise the faecal microbiome and volatile metabolome of Thoroughbred racehorses in training. Metabolomic characterisation was achieved using thermal desorption-gas chromatography-mass spectrometry (TD-GC-MS) and pyrosequencing of 16S rRNA genes was employed for taxonomic characterisation. The ability of these methods to detect change in metabolome and bacterial community structure following dietary intervention was also determined.

Materials and methods

Study population and sampling

Our study population comprised 8 castrated male, Thoroughbred racehorses between the ages of 4 and 6 years. Each horse was stabled at the same premises, under the management of the same trainer and in active flat-race training. All horses were free from concurrent medical treatment and were fed a standard diet comprising 1% body weight (bwt) fed as a mix of bruised oats, naked oats and oat balancer (ratio of 1:0.5:1 respectively, 13 MJ/kg bwt dry weight energy density), 1% bwt Timothy hay, 0.15% bwt alfalfa hay plus linseed oil, salt and electrolyte supplementation. Daily intake was divided between 3 feeds, the evening feed comprising 66% of each horse's daily concentrate (grain) intake.

All horses in this population had faeces sampled once before supplementation and 6 horses were available for repeat sampling (2 lost from study due to orthopaedic injury) after 6 weeks supplementation of their diet with amylase-rich malt extract[a]. Briefly, this is prepared by hot water extraction of maltose from barley, evaporation to an 80% sugar solution and conversion of starch to simple sugars by amylolytic enzymes which are present in excess [23]. The rationale for this supplement is that small intestinal amylase production is known to be low in the horse compared with other species [24] and exogenous dietary amylase previously has been shown to enhance equine carbohydrate digestion [25]. This supplementation was used in order to determine whether our analysis of faecal microbiome and volatile metabolome detected changes in the post supplementation samples. Amylase-rich malt extract (200 g) was added to morning and evening feeds with 70% of the daily dose being administered to the larger evening feed.

All sampling took place in the morning, immediately following exercise, approximately 3 h after the horses received a small meal. Horses were excluded from the study if they were receiving any concurrent medication or if they were unavailable for sampling. Sampling and metadata collection were carried out under University of Liverpool ethics approval RETH000363, with the informed consent of the trainer. Freshly voided faeces were collected into polyethylene sample containers and immediately frozen in liquid nitrogen prior to transfer into a freezer at -80°C.

Thermal desorption-gas chromatography-mass spectrometry (TD-GC-MS)

Frozen faecal samples were defrosted and 5 g of each sample placed inside a Nalophan sampling bag, made up of 65 mm diameter Nalophan NA tubing, 25 μm thick. One end of the bag was fitted with a Swagelok connector, the other was sealed and filled with hydrocarbon free air to generate the headspace of volatile organic compounds (VOCs). The bags were then placed in an incubator at 40°C for 1 h to allow the VOCs to equilibrate between headspace and solid sample. The Nalophan bags were connected to a thermal desorption (TD) tube for subsequent analysis by GC-MS to preconcentrate the headspace via an automated pump using 200 ml of faecal headspace. Standard stainless-steel TD sorbent cartridges were used, containing dual packing comprising 50% Tenax TA and 50% Carbotrap[b]. Cartridges were conditioned before use by purging with helium carrier gas for 2 min at room temperature followed by 1 h at 320°C.

Captured volatiles were analysed using an AutoSystem XL gas chromatograph equipped with an ATD 400 thermal desorption system and TurboMass mass spectrometer[c]. CP grade helium[d] was used as the carrier gas throughout, after passing though a combined trap for the removal of hydrocarbons, oxygen and water vapour. Cartridges were desorbed by purging for 2 min at ambient temperature then for 5 min at 300°C. Volatiles purged from the cartridge were captured on a cold trap which was initially maintained at -30°C. Once desorption of the cartridge was complete, the trap was heated to 320°C and maintained at that temperature for 5 min while the effluent was transferred to the gas chromatograph via a heated (180°C) transfer line.

A Zebron ZB624 chromatographic column[e] (dimensions 30 m × 0.4 mm × 0.25 mm ID) was maintained at 50°C for 4 min following injection and was then raised at 10°C/min to 220°C for 9 min. Separated products were transferred by heated line to the mass spectrometer and ionised by electron bombardment. The spectrometer was set to carry out a full scan from mass/charge ratios (m/z) 33–350 using a scan time of 0.3 s with a 0.1 s scan delay. The resulting mass spectra were combined to form a total ion chromatogram (TIC) by the GCMS integral software (TurboMass version 4.1) and resolved compounds were identified using AMDIS software and the NIST mass spectral database.

Univariate data analysis

Data were classified as presupplementation (NA) and post supplementation (NB). For all subjects GC-MS analytes were ordered by abundance. Descriptive statistics were generated for each group. Using data from the 6 horses with pre- and post supplementation samples, box and whisker plots were generated for the 8 most abundant compounds, grouped by classification and the significance of intergroup variation assessed by Wilcoxon rank sum test, a P value of less than 0.05 indicating a significant difference between group means.

16S rRNA pyrosequencing

Bacterial DNA was extracted from faecal samples using the QAIamp, detergent-based faecal DNA extraction kit followed by PCR of bacterial DNA with commercially available reagents[f]. The PCR amplification of the V1-V3 region of the 16S rDNA gene used fusion primers that included the 16S primer, sequencing adapters and multiple identity (MID) tags. The following primers were used: Forward primer (Primer A): 5′-CCATCTCATCCCTGCGTGTCTCCGACTCAG-{MID}-{template-specific sequence}-3′; and reverse primer (Primer B): 5′-CCTATCCCCTGTGT GCCTTGGCAGTCTCAG-{template-specific sequence}-3′. Sample-specific MID tags were used to multiplex and pool samples. Amplicon libraries were constructed using the Titanium amplicon kit[g]. Multiple independent PCRs were performed for each sample, combined and purified with Ampure[h] magnetic purification beads. No-template extraction controls were analysed due to the lack of visible products. Products were quantified using Quant-iT PicoGreen dsDNA assay on a Qubit[i] and a pool of purified PCR products was made so that there were equimolar ratios of up to 18 MID tagged samples per pool. The pooled products were sequenced using a Roche 454 FLX pyrosequencer at the University of Liverpool Centre for Genome Research.

Sequence data analysis

Sequences were split according to their barcode and analysed using QIIME [26]. 16S rRNA gene sequences were processed using the QIIME implementation of 'USEARCH' to filter noisy sequences and chimeric (*de novo* method) sequences, to normalise sequence numbers and perform OTU picking using a threshold of 97% pairwise identity on the set of de-multiplexed reads [27,28]. Other clustering and denoising algorithms were used but only USEARCH was able to fully process the data. Operational taxonomic units were classified taxonomically using the Ribosomal Database Project (RDP) classifier 2.0 [29]. Data was submitted to the NIH Sequence Read Archive, accession number PRJNA255136. Representative sequences from each OTU were aligned using PyNast [30] and a phylogenetic tree built using 'fasttree' implemented in QIIME. A table of OTU counts per sample was generated and used in combination with the phylogenetic tree to calculate alpha and beta diversity.

Rarefaction analysis was conducted using the QIIME scripts 'multiple_rarefaction.py' and 'alpha_diversity.py'. The QIIME metric 'observed species' was used to estimate alpha diversity in the dataset. Phylogenetic beta diversity estimates using weighted unifrac were implemented using QIIME scripts 'beta_diversity_through_plots.py' [31]. Linear Discriminant Analysis (LDA) effect size (LEfSe) was used to estimate the effect of each differentially abundant feature across the table of OTU counts per sample between pre- and post supplemented horses [32]. This method is robust in the face of multiple comparisons.

Results

Faecal volatile metabolome

Table 1 lists the identity of all endogenous metabolites detected by GC-MS from the faeces of our population pre- and post supplementation. Figure 1

TABLE 1: Prevalence of VOCs in faecal headspace (% horses positive) detected by GC-MS, for each group of horses

Headspace VOC	NA (n = 8)	NB (n = 6)
1,3-Dioxolane, 2-methyl-	100	100
2-Butanol	100	67
2-Butanone	100	100
Acetic acid	100	100
Acetone	100	100
Butanoic acid	100	83
Phenol, 4-methyl-	100	100
Propanoic acid	100	83
Tetradecane	100	50
2,3-Butanedione	88	100
2-Propenoic acid, 2-methyl-, methyl ester	88	67
Butanoic acid, 2-methyl-	88	67
Butanoic acid, 3-methyl-	88	67
Isopropyl alcohol	88	100
Pentanoic acid	88	33
Propanoic acid, 2-methyl-	88	67
R(-)3,7-Dimethyl-1,6-octadiene	88	100
Toluene	88	100
Butanal, 2-methyl-	75	100
Butanal, 3-methyl-	75	100
Disulfide, dimethyl	75	83
Pentane	75	100
1-Butanol	63	67
2,4-Dimethyl-1-heptene	63	50
Acetaldehyde	63	83
Dimethyl trisulfide	50	50
Hexanoic acid	50	0
Propanal, 2-methyl-	50	50
4 methyl heptane	50	0
1,6-Octadiene, 3,7-dimethyl-, (S)-	38	0
1-Propanol	38	100
Ethyl alcohol	38	100
Hexanal	38	67
Nonanal	38	33
Undecane	38	0
1-Pentanol	25	67
1-Propanol, 2-methyl-	25	33
2-Butanone, 3-hydroxy-	25	67
Benzaldehyde	25	67
Phenol	25	50
Nonane	25	0
Dodecane	17	0
Hexane	17	13
Hexane, 2,4-dimethyl-	17	0
Tridecane	17	0
2-Pentanone	13	0
2-Propenoic acid, 2-methyl-, 3-hydroxypropyl ester	13	0
Acetic acid, hydrazide	13	33
Dimethyl sulfone	13	0
Furan, tetrahydro-	13	17
Methacrolein	13	33
Octanal	13	0
Pentanal	13	33
Propanal	13	67
Styrene	13	0
2 methyl nonane	13	0
2 methyl pentane	13	17
2,3 dimethyl hexane	13	0
1,7-Octadiene, 2,7-dimethyl-	0	17
1-Butanol, 2-methyl-, (S)-	0	0
1-Butanol, 3-methyl-	0	33
1-Pentene, 2-methyl-	0	50
2-Butene	0	17
2-Propanol, 1,1,1,3,3,3-hexafluoro-	0	0
2-Propanol, 2-methyl-	0	67
3-Carene	0	0
á-Pinene	0	17
Benzene	0	17
Butanal	0	0
Decanal	0	17
Dimethyl sulfide	0	50
D-Limonene	0	0
Heptane, 4-methyl-	0	50
Propane, 2-ethoxy-2-methyl-	0	17
3 hexanone	0	17
3 methyl hexane	0	33
Decane	0	0
Heptane, 4-methyl	0	50
1,3-Dioxolan-2-one	0	17

NA = presupplementation racehorses, NB = post supplementation racehorses

illustrates the distribution of abundance measurements for the 8 most abundant compounds detected by GC-MS. For acetic and propanoic acid, median abundance is significantly reduced in faeces post supplementation compared with presupplementation ($P<0.05$). Abundance of butanoic, 3-methyl butanoic and 2-methyl propanoic acid is also reduced in post supplementation samples although the difference is not statistically significant.

Faecal microbiome

A total of 637,390 16S rRNA sequencing reads was obtained for 14 samples subjected to 454 pyrosequencing. The QIIME processing for all 14 samples resulted in 488,213 reads (75% of reads) post de-replication and quality control. These reads then underwent clustering and error correction which resulted in 183,453 clusters, this was reduced to 38,504 clusters after chimera removal. These 38,504 clusters represented 447,769 reads (70% of the starting number). A large number (17,598, 46%) of clusters are represented by singletons and double sequencing reads. Although these influence the estimations of Alpha diversity, ~50% of observed OTUs, they account for only 8% of total reads. This is demonstrated by the rarefaction analysis (Fig 2). Table 2 gives percentage abundance of taxa in pre- and post supplementation samples for pooled data.

Due to high levels of rare and low abundance OTUs, we used a cut-off rule whereby OTUs were considered to be 'verifiable' if they were represented by 10 reads (equivalent to >1% per sample) in more than 4 samples (i.e. must be seen between paired horse samples and in at least one other pair). This rule reduces the total number of OTUs to 4041. This reduced set was therefore used in all analysis other than alpha diversity calculations. Table 3 summarises the alpha diversity data using Chao1 – species richness, Shannon index and Simpson's index – species richness and abundance. Verifiable diversity for individual samples was in the range 1200–3000 OTUs.

The bacterial communities observed in the faeces of our Thoroughbred racehorse population post supplementation were dominated by Firmicutes (53%), Bacteroidetes (42%), with a ~5% contribution from other bacterial phyla (Table 2). Clostridiales, Actinomycetales, Lactobacillales and Bacteroidales were the most frequently observed of the 84 orders identified. Graphical representations of community structure pre- and post supplementation are presented in Supporting information Items S1 and S2. Novelty of the equine faecal microbiome is indicated by the large proportion of OTUs that cannot be identified at lower taxonomic levels (labelled 'other' in Supporting information Items S1 and S2).

Linear discriminant analysis (Fig 3) suggests that there are significant changes in the relative abundance of both Bacteroidetes and Firmicutes families, such as Prevotellaceae and Veillonellaceae, after the 6 week period of dietary supplementation.

Discussion

This study is the first to report both the volatile metabolome and taxanomic characterisation of faeces from Thoroughbred racehorses, including how these measures change in response to a specific dietary intervention. Key findings of this study are: 1) the huge diversity and novelty of the equine faecal microbiome, 2) marked changes in patterns of fermentation as measured by VOCs, most likely a response to dietary supplementation and 3) individual variation in response to dietary supplementation.

Faecal volatile metabolome

Unsurprisingly, the catalogue of VOCs detected in equine faecal headspace is dominated by acids, alcohols and ketones, most likely arising from bacterial digestion of carbohydrate including dietary fibre (Table 1). A major difference between the faecal VOC metabolome of the horse and man is the lower frequency of detection of sulphide and disulphide compounds. Dimethyl sulphide was infrequently detected in horse faecal headspace but was present in most human samples [33]. These observations suggest differences in sulphur metabolism between human and equine intestinal tracts.

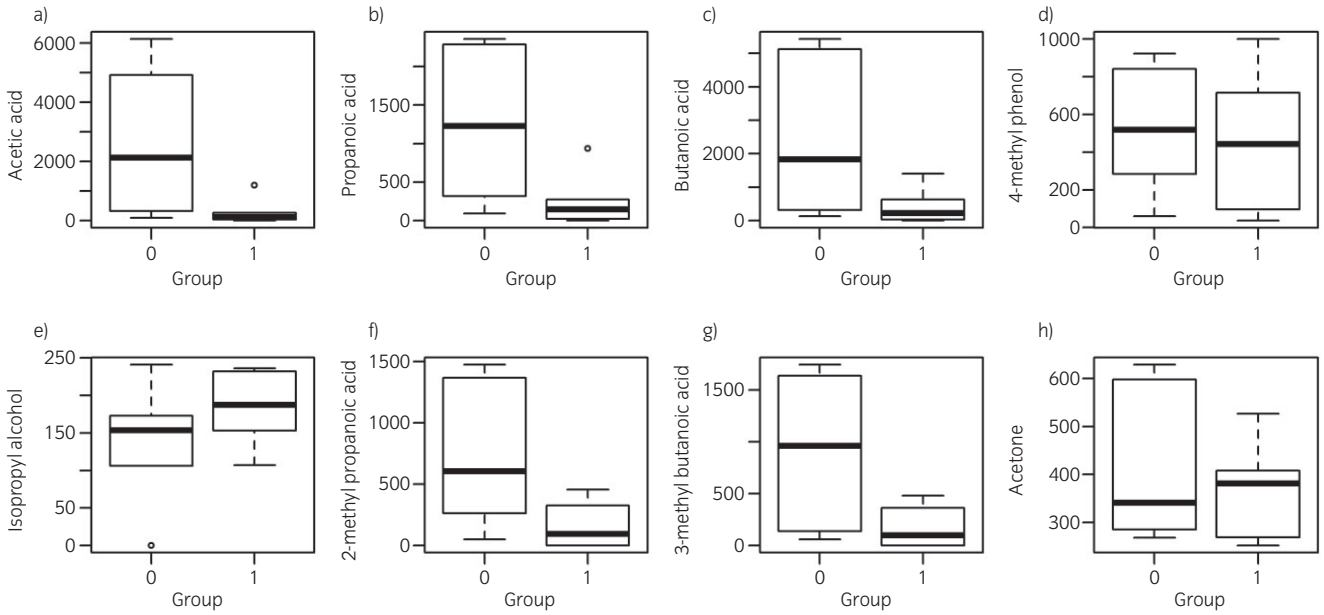

Fig 1: Change in abundance of 8 most abundant volatile organic compounds (VOCs) in faeces of 6 Thoroughbred racehorses following dietary supplementation with malt extract. Group identification: 0 = horses in race training presupplementation; 1 = horses in race training after 6 weeks supplementation. (a) acetic acid; (b) propanoic acid; (c) butanoic acid; (d) 4-methyl phenol; (e) isopropyl alcohol; (f) 2-methyl propanoic acid; (g) 3-methyl butanoic acid; (h) acetone. Abundance VOC expressed as ng/l. Difference between pre- and post supplementation abundance is statistically significant ($P<0.05$) for acetic and propanoic acid.

In contrast to human faecal headspace, a very limited range of aromatic compounds was identified. In human faecal headspace 56 different aromatics have been detected with many being present in 50% of samples or more [33]. The same study also reported detecting many alkenes including some with high frequency, e.g. limonene was found in nearly all samples from normal humans. Alkenes (2-butene, 3-carene, a-pinene and limonene) were only occasionally found in our equine samples.

Also of note is the marked difference in VOC diversity observed in our equine samples compared with human faecal samples. We detected 81 different VOCs in 14 different samples, Garner *et al*. reported 297 VOCs in 151 human samples [33]. A further difference is that in the human study 78 VOCs were present in 50% or more of the samples from nondiseased individuals; in our equine study only 28 VOCs fulfil this criterion. While differences exist in sampling method, sample size and method of VOC analysis, these observations suggest that a more limited repertoire of VOCs may be present in horse faeces.

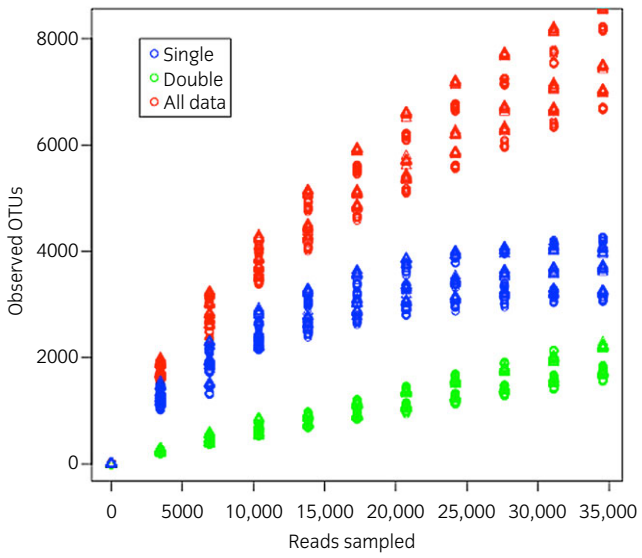

Fig 2: Rarefaction analysis of observed operational taxonomic units (OTUs) from faecal samples of 14 Thoroughbred racehorses. All data (red symbols) indicate a high level of taxanomic diversity, much of which is accounted for by single (blue symbols) or double (green symbols) sequence reads. Triangles and rectangles show pre- and post treatment data, respectively.

TABLE 2: The percentage abundance of taxa described in faecal microbiome of Thoroughbred racehorses presupplementation (NA) and post supplementation (NB), determined by 454 sequencing

Taxon	NA	NB
Bacteria; Firmicutes; Clostridia	57.831%	51.480%
Bacteria; Bacteroidetes; other	18.420%	12.150%
Bacteria; Bacteroidetes; Bacteroidia	15.070%	29.425%
Bacteria; other; other	5.410%	4.300%
Bacteria; Firmicutes; other	1.785%	0.800%
Bacteria; TM7; TM7_genera_incertae_sedis	0.348%	0.242%
Bacteria; Synergistetes; Synergistia	0.183%	0.016%
Bacteria; Bacteroidetes; Sphingobacteria	0.169%	0.923%
Bacteria; Firmicutes; Bacilli	0.167%	0.219%
Bacteria; Proteobacteria; Deltaproteobacteria	0.135%	0.147%
Bacteria; Firmicutes; Erysipelotrichi	0.133%	0.116%
Bacteria; Actinobacteria; Actinobacteria	0.124%	0.061%
Bacteria; Proteobacteria; other	0.069%	0.041%
Bacteria; SR1; SR1_genera_incertae_sedis	0.051%	0.012%
Bacteria; Proteobacteria; Epsilonproteobacteria	0.030%	0.015%
Bacteria; Proteobacteria; Gammaproteobacteria	0.019%	0.013%
Bacteria; Chloroflexi; Anaerolineae	0.018%	0.014%
Bacteria; Verrucomicrobia; Verrucomicrobiae	0.011%	0.002%
Bacteria; Proteobacteria; Betaproteobacteria	0.009%	0.012%
Bacteria; Tenericutes; Mollicutes	0.005%	0.004%
Bacteria; Fibrobacteres; Fibrobacteria	0.004%	0.005%
Bacteria; Bacteroidetes; Flavobacteria	0.003%	0.001%
Bacteria; Proteobacteria; Alphaproteobacteria	0.002%	0.001%

TABLE 3: Species richness, abundance and evenness metrics for a) the whole dataset and b) the data after the removal of operational taxonomic units with less than 10 reads in less than 4 samples

	Chao1	Observed species	Shannon diversity index	Simpson's index diversity 1–D
a)				
NA1	12,889	10,538	11.10	1.00
NA3	10,023	7634	11.21	1.00
NA4	9662	7861	10.75	1.00
NA5	8638	7222	10.14	0.98
NA6	3948	2438	9.78	0.99
NA7	4651	2956	9.72	0.99
NA8	4619	2636	10.06	1.00
NB1	4711	2740	10.21	1.00
NB3	6790	4038	10.86	1.00
NB4	11,277	9484	10.92	1.00
NB6	9897	7870	10.53	0.99
NB7	9463	7729	11.15	1.00
NB8	12,011	9953	11.42	1.00
b)				
NA1	3409	2970	9.48	0.99
NA3	3112	2532	9.59	1.00
NA4	2658	2237	9.20	1.00
NA5	2578	2116	8.15	0.96
NA6	1804	1231	8.64	0.99
NA7	2038	1378	8.52	0.99
NA8	2024	1308	8.91	0.99
NB1	2156	1376	9.04	0.99
NB3	2838	1911	9.70	1.00
NB4	2918	2448	9.13	0.99
NB6	2863	2384	8.86	0.99
NB7	2741	2276	9.31	1.00
NB8	3084	2707	9.63	1.00

In our Thoroughbred racehorse population faecal VOC measurement was a sensitive discriminator between faeces from different sampling points. Notwithstanding the limitations of our study (discussed below) we believe that the change in faecal volatile metabolome that we describe is most likely associated with dietary supplementation. Univariable analysis of GC-MS data indicates a decrease in many of the products of carbohydrate digestion at the second sampling point, following supplementation with amylase-rich malt extract. Some of the most abundant volatile components of the faecal metabolome were sensitive to dietary supplementation. We believe that the observed decrease in short chain fatty acids (SCFAs) is consistent with enhanced precaecal digestion of starch in horses receiving the supplement, leading to decreased carbohydrate reaching the caecum and being available for microbial fermentation [34]. While the present study is unable to directly characterise changes in the caecal metabolome and microbiome resulting from our dietary supplementation that is active in the small intestine, we believe that we can measure the downstream effect in faecal samples. This phenomenon has been reported in human studies [35]. The potential importance of precaecal carbohydrate digestion in human and animal intestinal health has been highlighted by one of the authors [36].

The biological significance of the observed changes in faecal metabolome is unknown. There is much evidence for the beneficial effects of SCFAs in the mammalian colon; butyrate is the preferential energy substrate for colonocytes, it also inhibits proinflammatory cytokine effects and SCFAs are involved in cell cycle regulation and induction of apoptosis [37]. However, the beneficial pharmacological and nutritional influences of SCFAs are balanced by the adverse mechanical effects of excess gas production [38,39]. Similarities between irritable bowel syndrome in man, often characterised by gaseous bloating and colic in the horse have been previously proposed by one of the authors [5]. More detailed studies of colonic VOC physiology are necessary to understand when physiological production of SCFAs becomes excessive and detrimental.

Faecal microbiome

The number of unique OTUs (sharing ≥97% nucleotide sequence identity) from human faeces has an upper range of 2000 OTUs [40] whereas in horses the observed range in our study (using the same methods as reference 40) is between 2500 and 10500 OTUs. Further analysis suggests that diversity in the horse gut is driven by the large number of singleton OTUs present in the horse gut communities. Adjusting the analysis for very low abundance OTUs resulted in a verifiable OTU range of 1200–3000. It is interesting to note that about 2000 OTUs were shared by 50% of horses and only 67 OTUs were common to all samples. Alpha diversity estimates are very sensitive to sequencing errors and chimeras in the data, both of these sources of error have been accounted for in the processing and analysis of our read data using USEARCH software. Even with the removal of such errors the OTUs should not be considered species as a 97% cut-off/clustering value will not be appropriate for all species in the community as evolutionary rates will differ between groups.

The high level of very low abundance bacterial populations is intriguing and not observed in man. It is possible that these bacteria are 'visitors' to the horse's intestinal lumen, travelling on the large volume of plant fibre, plus soil contamination, ingested daily by the horse. Such high frequency, low abundance microbial populations may represent environmental sampling by the horse; the functional importance of which is unknown.

This is the first description of the faecal microbiome of a phenotypically distinct population of Thoroughbred racehorses fed a high-energy diet. At the phylum level of classification we report 53% Firmicutes, 42% Bacteroidetes (5% Other) after supplementation. This is consistent with a previous Swedish report of healthy horses eating a concentrate diet [12]. A healthy, North American Quarter Horse population [41], fed a pelleted ration, was reported to have a Firmicutes:Bacteroidetes ratio of 70%:6% with Verrucomicrobia being the second most prevalent phylum. This marked variation in Firmicutes:Bacteroidetes ratio may arise from

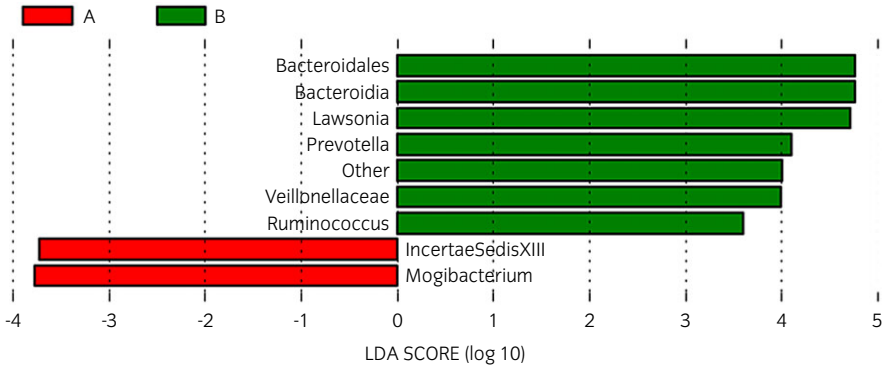

Fig 3: Linear discriminant analysis (LDA) indicating significant differences in relative abundance of 9 bacterial genera between pre- and post supplementation faecal samples (n = 6). Red bars indicate greater abundance presupplementation, green bars indicate greater abundance post supplementation.

experimental artefact or bias (e.g. differences in sample processing), dietary differences or geographical variation (Western European vs. North American).

Some other interesting taxonomic differences are apparent between our study and those previously published. We report Verrucomicrobia comprising only 0.05% of the faecal microbiome of our study population. Other studies report 18% in healthy horses, 28% in laminitics [41] and 4% in grass-fed horses [16]. The latter study also reports 2% Spirochaetes compared with 0.002% in our study and 1.8% TM7 (uncultivated bacterial group) compared with 0.5% in our study. Such differences may result from amplification or sequencing bias or may be genuine reflections of different bacterial community structure. More sequence data from phenotypically distinct populations of horses, fed on well-characterised diets, is required to further interpret the interplay between diet and the horse intestinal microbiome.

Although very marked changes in VOC profile were recorded after supplementation with amylase-rich malt extract, changes in bacterial community structure were relatively small and inconsistent between horses. From this we conclude that: 1) the dietary intervention in this study induced metabolic adaptation of existing bacterial communities rather than dramatic changes in community structure and 2) our data illustrates interhorse variation in response to dietary change. This later finding is reminiscent of the suggested existence of 'enterotypes' in man [42], but much larger populations of horses will need to be studied to explore this possibility fully.

The significant changes in faecal microbiome that we have associated with dietary supplementation are within some of the lower abundance taxa of the faecal microbiome. Increase in the Veillonellaceae (lactate-utilising bacteria) is potentially a beneficial response to increased dietary carbohydrate as they will consume lactate arising from increased hindgut fermentation, thus buffering pH changes. In a previous study [43], we observed no compensatory increase in abundance for this family of bacteria in response to increasing concentrate in the diet and increasing colonic lactic acidosis. We speculate that undetermined luminal environmental thresholds are operating to allow expansion of the Veillonellaceae only within a certain 'window.' The functional implications of the observed increase in abundance of an uncharacterised Bacteroidales family, Lawsonia, Prevotella and Rumenococcus and the decrease in abundance of Incertaesedis XIII and Mogibacterium are unknown at present. The functional importance of genera with unchanged abundance is also unknown.

This study used a highly homogeneous population of horses, managed in a very standardised fashion. While this strategy is useful to control variation within the study population, it does limit the extent to which our results should be extrapolated to other populations managed differently. Our study did not use a crossover design, we did not study a control group of horses that received no dietary supplementation for the duration of the study nor did we undertake repeated sampling over time. It is possible that the differences in metabolome and microbiome that we observed between sampling points were associated with unmeasured variables that changed during this time. The risk of such a confounding effect was minimised by highly standardised feeding and management regimens. A further limitation of this study is the use of faeces as a biological sample. While this is a highly convenient sample to collect, microbial communities in the faeces are clearly different from those in the more orad large colon and caecum [12].

As our knowledge of the horse intestinal microbiome and associated metabolome increases, there is a need to understand the sources of variation observed. There is apparent variation in faecal microbiota between different breeds of horse; is this a consequence of diet, geographical location or inheritance of maternal microbiota? Our study also suggests individual variation between horses of similar breed receiving a very similar diet. This phenomenon is currently the subject of investigation in man and the existence of 3 core 'enterotypes' has been suggested [42]. There is also a need for metagenomic studies that characterise the functional response of the horse microbiome to dietary intervention. The present study indicates that large-scale shifts in bacterial populations do not occur in spite of profound changes in metabolome reflecting marked functional change. Further population-based studies are required to understand the complexity of the equine intestinal microbiome and associations with health and disease.

Authors' declaration of interests

No competing interests have been declared.

Ethical animal research

Sampling and metadata collection were carried out under University of Liverpool ethics approval RETH000363, with the informed consent of the trainer.

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Authorship

The study was conceived and designed by C.J.Proudman, J.O. Hunter and E.E. Escalona; sampling was undertaken by C.J. Proudman and E.E. Escalona. Metabolomic analysis was performed by C. Turner and C. Batty, sequencing data analysis was undertaken by A.C. Darby. All authors contributed to data analysis and interpretation and to manuscript preparation.

Manufacturers' addresses

[a]Muntons, Stowmarket, Suffolk, UK.
[b]Markes International Limited, Llantrisant, UK.
[c]Perkin Elmer, Wellesley, Massachusetts, USA.
[d]BOC Gases, Guildford, Surrey, UK.
[e]Phenomenex, Torrance, California, USA.
[f]Velocity DNA kit, Bioline, London, UK.
[g]454 Life Sciences, Roche Diagnostics, Burgess Hill, West Sussex, UK.
[h]Agencourt, High Wycombe, Buckinghamshire, UK.
[i]Life Technologies, Paisley, UK.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Item S1: Characterisation of the faecal metabolome and microbiome of Thoroughbred racehorses. Pie chart representing the identification and abundance of faecal microbiota at various taxonomic levels in 8 Thoroughbred racehorses presupplementation.
Item S2: Characterisation of the faecal metabolome and microbiome of Thoroughbred racehorses. Pie chart representing the identification and abundance of faecal microbiota at various taxonomic levels in 6 Thoroughbred racehorses post supplementation.