"Bowel-on-the-bench:" proof-of-concept of a three stage, in vitro, fermentation model of the equine large intestine

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Abstract The intestinal microbiota of the horse, an animal of huge economic and social importance worldwide, is essential to the health of the animal. Understanding the intestinal ecosystem and its dynamic interaction with diet and dietary supplements currently requires the use of experimental animals with consequent welfare and financial constraints. Here, we describe the development and assessment, using multiple analytical platforms, of a three vessel, continuous flow, *in vitro* model of the equine hindgut. After inoculation of the model with fresh horse feces, bacterial communities established in each vessel that were taxonomically similar to those in the source animal. Short chain and branched chain fatty acid (SCFA/BCFA) production within the model at steady-state was consistent with expected bacterial function although higher concentrations of some SCFA/BCFAs, were apparent relative to that of *ex vivo* gut content. We demonstrate inter-model repeatability and the ability of the model to capture some aspects of individual variation in bacterial community profiles. This proof-of-concept study, including recognition of model limitations, supports its future development as a tool for investigating the impact of disease, nutrition, dietary supplementation and medication on the equine intestinal microbiota.

Importance The equine gut model that we have developed and described has the potential to facilitate the exploration of how the equine gut microbiota is affected by diet, disease and medication. It is a convenient, cost-effective and welfare-friendly alternative to *in vivo* research models.

INTRODUCTION

Commensal bacteria that reside within the large intestine of the equine gastrointestinal tract are vital for the horse to be able to utilise a forage-based diet. Some of these

bacteria produce short chain fatty acids (SCFAs), which are absorbed through the gut wall and contribute to the energy requirements of the horse. Sequencing of bacterial 16S rRNA genes from gastrointestinal content or feces has been used to profile the gut microbiota of healthy horses (1–5) and to assess the impact of diseases such as colitis and grass sickness on equine fecal bacterial communities (6,7). In common with the majority of human microbiota studies, most disease-related studies have characterized the equine fecal microbiota due to ease of access to samples. Equine feces have been shown to be broadly representative of bacterial communities within distal parts of the large colon (1) and to more orad parts of the intestinal tract.

To accurately analyze bacterial communities within the gastrointestinal tract of the horse, direct sampling of each region of interest would be ideal. While this can be done *post-mortem* or with fistulated horses, both techniques have limited application due to financial, ethical and welfare implications. Therefore, a fermentation model representing the microbiota of the equine large intestine *in vitro* would facilitate this important area of equine health research, enhancing our ability to explore the impact of diet, pathogens, novel foodstuffs, dietary supplements and drugs on the equine gut microbiota.

Models of the human gut have been a valuable and widely used research tool (8). Studies have shown how an intervention with galactooligosaccharide (GOS) has similar effects on the microbial community *in vitro* when compared to in a human study population (9). *In vitro* and *in vivo* approaches were taken to study the effect of the prebiotic GOS on the gut microbiota of children with autism (10,11). Both approaches demonstrated an increase in butyrate production (in the gut model and human feces respectively) suggesting that the model is metabolically valid. Gut models have

provided preliminary insights on the effects of dietary supplement or drug on the human gut microbiota before commencing costly human trials.

In vitro fermentation models developed to represent bacterial activity in the equine gastrointestinal tract have been reported previously (12–20). However, the majority of these models have used a simple medium or equine feed to maintain fecal bacterial populations and do not replicate the continuous flow of ingesta through the gastrointestinal tract (12,13,16–22). These models often use gas production as a marker of bacterial function and measure the kinetic properties of the bacterial populations cultured (13,17–21). Sequencing of bacterial DNA from fermentation samples allows for a more comprehensive overview of the bacterial communities present, however this approach has so far only been reported in one equine fermentation study (14).

In this study, we report on the development and performance of a three-stage fermentation model designed to simulate bacterial communities in the equine large intestine. Two experiments were designed: i) a concordance study of three individual gut models, each inoculated with feces from a different horse (*post-mortem*), to evaluate the extent to which the gut bacterial profiles of individual horses are replicated, and ii) a repeatability study of two gut models inoculated with feces from the same horse (*pre-mortem*). To analyse samples taken from the vessels of the gut model we used fluorescence *in situ* hybridization (FISH) analysis to assess total bacterial numbers and 16S rRNA bacterial gene sequencing to characterize the microbial community profile *in vitro*, compared to the samples taken *ex vivo*. Proton nuclear magnetic resonance (^{1}H NMR) spectroscopy and gas chromatography were used to measure a broad range of metabolites to evaluate the functional activity of the model microbiota. Specifically we report on i) model concordance with *in vivo* gut

bacterial populations, ii) repeatability of the model, and iii) the ability of the model to capture individual variation in bacterial community profile.

RESULTS

Gut model medium was adapted to reflect a normal equine diet

The acid detergent fibre (ADF) and starch content of gastrointestinal content samples were analyzed to inform the compositional changes made to the gut model media. We aimed to replicate the diet of the average UK leisure horse in the media used to feed the model and the horses sampled to inoculate it. A large percentage of the gastrointestinal content of the equine ileum, large colon and feces was acid detergent fibre (ADF, Figure S1). Feces had the highest percentage of ADF (42.9 % ± 3.9 %) and the lowest percentage of starch (0.56 % ± 0.49 %), whereas the ileal content had the lowest percentage of ADF (25.9 % ± 10.8 %) and the highest percentage of starch (3.8 % ± 3.3 %). The analysis of ADF and starch from ileum content informed the cellulose and starch content of the gut model media used to feed the model.

The equine *in vitro* gut model can support a metabolically functioning bacterial population

The concentrations of five short chain and branched chain fatty acids (SCFA/BCFA) produced in each vessel of the *in vitro* gut models were measured during the concordance study to assess the metabolic output of bacterial fermentation. Figure 1 shows the concentration of acetate, propionate, butyrate, isobutyrate and valerate in the three vessels, as a mean of the three separate gut models. Concentrations of

117 acetate and propionate rose quickly in all three vessels after initial inoculation (Figure
118 1A and B). Concentrations of butyrate, isobutyrate and valerate only increased
119 marginally between T-1 and T0, but then increased sharply after the flow of media
120 wasstarted at T0 (Figure 1C, D and E). The mean concentration for all SCFA/BCFA
121 measured was observed to stabilise in all three vessels by T5 as there was no
122 significant change in concentration between T3 and T5 ($p > 0.05$).

123 The metabolic profiles of the three gut models in the concordance study were
124 compared to that of the gastrointestinal content of the donor horses. Figure 2 shows
125 the PCA model ($R^2 = 0.64$) built with metabolic data from gastrointestinal contents,
126 feces from the rectum and all samples taken from the three gut models. The PCA
127 scores plot shows that the largest source of variation within the dataset (PC1,
128 representing 50 % of the total variation the dataset) was explained by samples taken
129 from the model after T1 having higher concentrations of acetate (Figure 2A and B).
130 Within the second principle component (PC2) of this PCA model samples of
131 gastrointestinal content clustered away from samples from the gut models
132 (representing 15 % of the total variance, Figure 2A). This was due to the content of
133 the large colon having higher concentration of acetate compared to the gut model
134 (Figure 2C).

135 A PCA model was constructed with the metabolic profiles of the gastrointestinal
136 content and samples taken from the three concordance study models at T5 ($R^2 =$
137 0.64). There was separation of points representing samples from the two groups in
138 PC1 (explaining 48 % of the variance in the dataset) in the PCA scores plot (Figure
139 S2A) indicating metabolic differences between samples of gastrointestinal content and
140 gut model. When these differences were explored further, samples of gastrointestinal
141 content had higher concentrations of acetate whereas the samples from the gut model

at T5 had higher levels of valerate and propionate (Figure S2B). An OPLS-DA model was also built with the metabolic spectra from these two groups of samples (Q^2Y = 0.82). This supervised model showed the differences in levels of acetate, propionate and valerate (also evident in the PCA model) alongside higher levels of diethylene glycol (DEG), ethanol, trimethylamine (TMA) and iso-valerate in samples from the gut models (Figure S2C).

The equine gut model can replicate part of the equine large intestinal microbiota

An estimate of total bacteria was created using FISH analysis to assess bacterial concentration within the gut model after fecal inoculation. Mean total bacteria for the three gut models of the concordance study were lower in the vessels of the gut model at T-1 (2.68×10^8, 2.86×10^8, 2.21×10^8 bacteria per ml) compared to gastrointestinal content (3.93×10^8, 3.40×10^8, 3.32×10^8 bacteria per ml). At T0 bacterial counts had risen in all three vessels (2.81×10^8, 4.02×10^8, 3.69×10^8 bacteria per ml). By T5 (steady state) bacterial proliferation had plateaued, with vessel one having the highest counts (5.51×10^8 bacteria per ml) and vessel three the lowest (3.74×10^8 bacteria per ml). Bacterial alpha diversity (measured as observed OTUs) within the gut models at T0 and T5 was significantly lower than the bacterial diversity of gastrointestinal content and the models at T-1 ($p < 0.05$, Figure S3A)

Unsurprisingly bacterial community profile at phylum level of the model at T-1 (immediately after inoculation) was similar of that of gastrointestinal content of the horses that provided feces for inoculation (Figure 3). However, the abundance of *Verrucomicrobia* was greater in the *in vitro* gut models immediately after inoculation (T-1) than in the gastrointestinal content (14 % vs 1 %, $p < 0.05$). After the gut models

were batch-cultured for 24 hours (T0) an increased abundance of *Proteobacteria* (from an average of 2 % to 27 %) and *Firmicutes* (from an average of 40 % to 64 %) was observed in all vessels ($p < 0.05$ from T-1 to T0 for both bacterial phyla). However, by T5 the relative abundance of *Firmicutes* and *Bacteroidetes* was comparable to that of the gastrointestinal content; and the relative abundance of *Proteobacteria* was much reduced relative to T0 (from an average of 37% to 8%, $p < 0.05$).

On average, samples taken directly from the cecum were composed of 40 % *Bacteroidetes* and 46 % *Firmicutes* (as a mean of the three horses sampled). Whereas, in the gut model these proportions of bacteria were 21 % and 59 % (as an average for vessel 1 of the three models of the concordance study), respectively. For right ventral colon the proportions were 40 % and 47 % for the gut contents and 15 % and 53 % in the gut model (vessel 2), respectively and for the left ventral colon the gut contents proportions were 37 % and 50 % and gut model proportions 25 % and 48 %, respectively (vessel 3).

Interestingly, the percentage of the bacterial community within the *in vitro* gut model that was identified as *Bacteroidetes* decreased further down the model (from vessel 1 to 3), similar to the changes observed in aboral regions of the large colon. There were higher levels of *Bacteroidetes* in vessel three of model one (M1V3), than in vessel three of the other two models (M2V3 and M3V3). There was a higher relative abundance of *Synergistetes* in the gut model at T5 (mean of all three vessels = 14 %) compared to the gastrointestinal content (< 1%). There were also slight differences in bacterial communities of the three models (M1, M2 and M3), inoculated with feces from different horses. M2 had a significantly higher percentage of reads identified as *Firmicutes* compared to M3 ($p < 0.05$) and M3 had a significantly higher percentage of *Verrucomicrobia* and *Synergyistetes* bacteria than the other two models ($p < 0.05$).

The mean SCFA/BCFA concentration and bacterial phyla count for each vessel of the concordance study gut models were used to build regression models. A number of the models built showed a positive correlation between SCFA/BCFA and bacterial phyla with good model fit ($R^2 = 0.5$). However only a few of these associations were significant ($p < 0.05$) these included: *Fusobacteria* and acetate (V1), *Synegistetes* and valerate (all vessels) and *Synegistetes* and isobutyrate (V1 and V3). For R^2 and *p* values for regression analyses see Table S2.

Bacterial community profiles were also illustrated at class and order level (Figure S4). The bacterial class *Clostridia* (on average 47 % in gastrointestinal samples and 53 % average in T5 gut model samples) and order *Clostridiales* (47 % and 53 %) were at the same percentage abundance as the *Firmicutes* phylum (48 % and 53 %) and the class *Bacteroidia* (37 % and 21 %) and order *Bacteroidales* (37 % and 21%) were at the same percentage abundance as *Bacteroidetes* phylum (39 % and 21 %) in all gastrointestinal and gut model samples. Differences associated with differing relative abundance of *Clostridia/Clostridiales* and *Bacteroidia/Bacteroidales* were observed between the gut models inoculated with different feces.

Venn diagrams were produced to visualise the number of distinct operational taxonomic units (OTUs, bacterial groups with different taxonomic classification) that were shared between the three vessels of the gut models (at T5) of the concordance study and their corresponding region of the equine large intestine (Figure 4). Vessel 3 shared the highest number of OTUs, with 57 OTUs (59 % of the total OTUs identified in samples from vessel 3) being common between vessel 3 and content from the left

214 ventral colon. Vessel 2 shared 53 OTUs (56 %) with the right ventral colon samples
215 and vessel 1 shared 47 OTUs (57 %) with cecum samples.

216

217 **The metabolic and microbial profile created by the equine gut model is**
218 **repeatable.**

219 The concentration of the five SCFA/BCFA measured using gas chromatography
220 significantly rose between T-1 and T1 in all of the vessels of the two gut models of the
221 repeatability study, before plateauing ($p < 0.05$, Figure 5). As with the concordance
222 study, the models from the reproducibility study were deemed to be stable by T5 ($p >$
223 0.05 for all SCFA/BCFA between T3 and T5). The concentration of butyrate and
224 valerate slightly decreased after steady state was reached (between T5 and T7), but
225 this was not significant ($p > 0.05$). Furthermore, the concentration of the five
226 SCFA/BCFA measured by gas chromatography was not significantly different in the
227 two models of the repeatability study ($p > 0.05$ for all SCFA/BCFA at all turnovers).

228 The reproducibility of the metabolic signature of the two gut models was
229 assessed by building a PCA model with the ^{1}H NMR spectra gained from the two gut
230 models of the repeatability study ($R^2 = 0.75$, Figure S5). The largest source of variation
231 within this dataset (PC1, representing 63 % of the total variance in the model) was the
232 increase in concentration of propionate and acetate over the first four days of the
233 model (T-1 to T1). Points representing samples from the two different models clustered
234 together after T1, indicating that the two models were metabolically similar.

235 Total bacteria counts calculated by FISH were similar in the two gut models of
236 the repeatability study (M4 and M5). M4 had slightly more bacteria in all three vessels
237 at T5 compared to M5 (mean bacteria per ml for M4 was 4.02×10^8 and M5 was 3.88

238 x 10^8), but this was not statistically significant ($p > 0.05$). There was a slight increase
239 in total bacteria between T5 and T7 in all of the vessels of the two gut models of the
240 repeatability study (on average the total bacteria of all vessels increased by 3.74 x
241 10^7), but this was not statistically significant ($p > 0.05$).

242 The bacterial alpha diversity (measured as observed OTUs) within the models
243 at T0, T5 and T7 was significantly lower than the bacterial diversity of the fecal sample
244 used as inoculum and the model at T-1 ($p < 0.05$, Figure S3B). However, bacterial
245 diversity in the models did not significantly change between T5 and T7 ($p > 0.05$).
246 Bacterial community profiles were constructed for the two gut models of the
247 repeatability study to assess reproducibility of the equine gut model (Figure 6). There
248 were no significant differences between the two models when the bacterial phyla
249 identified in each of the three vessels at models at T5 were compared ($p > 0.05$). There
250 were also no significant differences between the bacterial phyla profile of each vessel
251 at T5 and T7 ($p > 0.05$). Bacterial community profiles at class and order level were
252 similar between the two models of the repeatability study and stable between T5 and
253 T7 (Figure S6).

254

255 **DISCUSSION**

256 We have presented a description of an *in vitro* model of the equine hindgut and have
257 examined it against intestinal content samples taken from euthanized horses. This
258 allowed for the assessment of the equine gut model's ability to maintain bacterial
259 communities representative of that found *in vivo,* the repeatability of the gut model
260 setup and the model's ability to capture individual variation that exists in the gut
261 microbial communities of horses. Models, by definition, are not exact representations

of reality. We acknowledge that a model of the equine microbiota will never be an exact replica of the real bacterial community within the equine large intestine. The model will however replicate aspects of the microbial community to allow interrogation and experimental manipulation in a more convenient and less invasive way. Data presented in this paper demonstrate the strengths and limitations of this novel gut model system and its potential to extend our understanding of equine intestinal health.

The three equine gut models of the concordance study showed that the fecal bacteria introduced to the gut model were able to metabolize the gut model media and produce SCFA/BCFA. Immediately after inoculation with feces, the abundance of acetate and propionate within the model increased sharply. Butyrate, isobutyrate and valerate were not observed to notably increase over the first 24 hours. However, these three SCFA/BCFA rose sharply in all three vessels during the first three days of being fed with media. Differences in the timing of SCFA/BCFA appearance may reflect differential acclimatization of the fecal bacterial communities to the gut model environment; bacterial communities producing acetate and propionate adapting to the environment more rapidly than those producing butyrate, isobutyrate and valerate. By T5, production of all five SCFA/BCFA had stabilized and the models were deemed to have entered “steady state”. In human gut model studies this is the point at which a food supplement or drug intervention is added to be studied (10,23–26).

Multivariate statistical models were used to identify metabolic differences between gastrointestinal content and the gut models. Ethanol, valerate, iso-valerate, propionate, DEG and TMA were at higher concentrations in the gut model at steady state compared to gastrointestinal content. Choline can be degraded by the gut microbiota to liberate ethanol and TMA. Ethanol can also be produced via the metabolism of keto acids and saturated fatty acids following the microbial breakdown

of amino acids. Similarly, iso-valerate arises from the microbial degradation of leucine. These findings indicate that the functional capacity of the equine gut microbiota to liberate amino acids from ingesta is preserved in the gut model system. The accumulation of theses metabolites is most likely due to the lack of absorption in the vessels of the gut model system. However, acetate was present in lower amounts in the equine gut model compared to gastrointestinal contents. Acetate is a product of bacterial carbohydrate fermentation and is usually absorbed by the gut wall so it can undergo further metabolism to generate ATP. Acetate concentrations in V1 of the concordance study models were found to be significantly positively correlated with the presence of known acetate producing bacterial phyla*, Fusobacteria* (27,28). Lower levels of acetate in the gut model suggest that carbohydrate availability may be less in the gut model or that some of the carbohydrate-fermenting bacteria cannot be supported. Alternatively, the acetate produced could be washed through the gut model system into the waste by the constant flow of media. The high levels of acetate in gastrointestinal content emphasises the importance of acetate-generating bacteria to enable horses to liberate energy from their diet.

FISH analysis of samples of gastrointestinal content and the vessels of the concordance study gut models provided an estimate of the number of total bacteria in the two sample types. The mean concentration of bacteria in vessel one was higher than that seen in the cecum, but this was also the vessel in which total bacteria varied the most between the three gut models from the concordance study. The differences in total bacteria between the three vessels was similar to that seen in the three sample sites of the large intestine although they are more pronounced in the gut model. This may be due to the progressive nutrient depletion as media passes through the model system.

We chose to use 16S gene sequencing for taxonomic characterisation of the bacterial communities present in the model. The advantages of this method are efficiency and breadth of coverage but we also recognise the disadvantage of limited resolution and specificity. A more balanced approach for future studies might include the use of quantitative PCR for targeted taxonomic (and functional) markers of interest. Future characterisation of this model system could also include evaluation of the virome and mycobiome.

Bacterial community profiles of the gastrointestinal content samples that we used were consistent with those reported previously (5) and dominated by *Bacteroidetes* and *Firmicutes*. Immediately after inoculation all three vessels of the gut models had a similar composition to fecal samples. After the first 24 hours of fermentation between T-1 and T0, there is a shift in the bacterial populations, where *Proteobacteria* and *Firmicutes* become dominant. However, by T5 the percentage of *Firmicutes* and *Bacteroidetes* bacteria in the three vessels did not significantly differ from the equivalent regions of the large intestine and the initial *Proteobacteria* overgrowth was reduced.

It is worth noting, however, that not all the populations present in gut content samples were restored in the model on reaching steady state. This situation will be largely dictated by the diet of the horse providing samples for inoculation of the model system, as compared to the nutrients available within the model media. Varying the carbon and nitrogen sources in the media could aid the growth of a more diverse range of organisms. Change in bacterial community dynamics have been observed in human gut models by varying media composition and model retention time (8). In equine and human gut model systems, the media flowing through the model means that different nutrient availability occurs in different regions, enabling different environmental

337 conditions to be modelled, supporting growth of different microbial populations at
338 different enteric sites.

339 The bacterial profile for vessel three of model one (M1V3) seen in Figure 3 had
340 a higher percentage of *Bacteroidetes* than expected; the percentage of *Bacteroidetes*
341 usually decreases from vessel two to vessel three (as can be seen in M2 and M3).
342 This high level of *Bacteroidetes* is likely to relate to the starting bacterial community in
343 this horse, as all models were treated identically, except for inoculation with feces from
344 different horses. The ability of this *in vitro* system to replicate aspects of bacterial
345 community profile representative of the equine large intestine is consistent at phylum,
346 class and order level.

347 Many of the low abundance bacterial taxa identified in the gastrointestinal
348 content were absent from the gut model. Significant differences were found when
349 comparing the OTUs identified in the gut models at T5 to the areas in the large
350 intestine that they represent. These OTUs may correspond to non-viable bacteria that
351 were detected in the gastrointestinal content and feces of horses, but were not able to
352 survive and proliferate in the gut model. Alternatively, it may reflect the failure of the
353 model to provide the exact environmental conditions needed to support the vast
354 number of low abundance bacterial groups normally found within the horse large
355 intestine. Clearly, a limitation of this model is that it does not support growth and
356 function of all the taxonomic diversity present in the large intestine of horses. To allow
357 a more diverse bacterial community to establish, other features of the equine large
358 intestine may need to be added to the model, such as epithelial cells and gut wall
359 secretions.By visualizing the unique OTUs that are shared between the gut model
360 vessels and the gastrointestinal content in a Venn diagram, we can see that the gut

models were able to maintain the bacteria that contribute to 39 – 43% of the unique OTUs identified in the gastrointestinal samples of horses.

Our studies have indicated that the equine gut model may capture some aspects of inter-individual variation seen in the gut microbiota of equine populations. Inoculating models with feces from different horse donors resulted in different bacterial community profiles by the time steady state is reached. This important aspect of the model is worthy of further investigation as it may allow the exploration of individual responses to food supplements and susceptibility to diseases that are mediated *via* the gut microbiota.

In the repeatability study two identical gut models were inoculated with feces from the same horse. SCFA/BCFA levels measured by gas chromatography showed that their production was similar in the two gut models of the repeatability study. ^{1}H NMR analysis of supernatants from these two models showed that metabolic profiles of the vessels were similar at all time points after T1. FISH analysis estimating the total number of bacteria maintained by the two models showed that differences in total bacteria between the three vessels were similar across the models. Bacterial DNA sequencing of samples taken from the three vessels of these two models at T5 showed comparable taxonomic profiles at both phylum and class level. Bacterial community profiles were stable for the two models between T5 and T7. The repeatability study provides preliminary evidence that models inoculated with the same fecal matter will produce microbial communities that are similar, both structurally and functionally.

We have reported the analyses used to assess the microbial and metabolic output of a three stage equine gut model. The model is metabolically functional and is able to support a bacterial community which replicates aspects of that found in the

equine large intestine. This model is not an exact replication of *in vivo* bacterial community of the equine large intestine, but does provide an *in vitro* alternative to studies that involve invasive or post-mortem sampling of horses. These may be particularly valuable for studying cecal microbiota, for which feces are not an adequate proxy. This model has the potential to aid in the understanding of how the equine gut microbiota is affected by diet, disease and drugs.

MATERIALS AND METHODS

Equine gastrointestinal content collection.

Samples of gastrointestinal content were obtained from horses, free of gastrointestinal disease, no more than three hours after euthanasia. The whole gastrointestinal tract was removed from the carcass and the large intestine identified. From each horse, two, 12 ml tubes were filled with content from the following areas: cecum, left ventral colon, right ventral colon and feces from the end of the small colon. Measurement of pH of fresh gut contents at all sample sites was achieved using a calibrated, hand-held pH monitor. Samples were transported on dry ice, then stored at -80 °C prior to analysis. Descriptive details of all horses sampled can be found in Table S1.

Compositional analysis of gastrointestinal content.

To inform the make-up of media ADF and starch content of gastrointestinal samples from three UK leisure horses (horse 1, 2 and 3 in Table S1) were analyzed. Samples were all free-dried to remove all moisture. ADF content of free-dried samples were analyzed using the filter bag technique (ANKOM technology). Starch content of

samples were analyzed by converting starch into glucose by treatment of the hot water extract with amyloglucosidase followed by acid hydrolysis (29). Total reducing sugars were measured colormetrically according to Fuller 1967 (30).

Three stage continuous culture system.

The human gut model developed by Macfarlane *et al.* 1998 (8) was adapted with the aim of replicating the microbiota of the cecum, right ventral colon and left ventral colon of the equine large intestine (Figure 7). This continuous culture system constituted of three vessels (V1, V2 and V3) with respective volumes of 300 ml, 400 ml and 200 ml to provide a scaled down version of their respective regions of the equine gastrointestinal tract. Temperature (at 38 °C by circulating water bath; Optima) and pH were automatically controlled (by Broadly James F695 pH probes and Fermac 260 pH measurement and control module; Electrolab) as described in Macfarlane and Eglyst (31). V1 and V2 were maintained at pH 6.3 - 6.5 and V3 at pH 6.6 - 6.8 to reflect the pH of the cecum, right ventral colon and left ventral colon respectively. All vessels and the media reservoir were stirred by magnetic fleas and maintained under anaerobic conditions by a continuous flow of anaerobic mix gas (80% N_2, 10% CO_2 and 10% H_2). A single channel, variable speed peristaltic pump (Electrolab) fed vessel one with media from the media reservoir at a rate of 900 ml every three days which represented the scaled flow rate of ingesta through the equine gastrointestinal tract. Media then flowed from vessel one to two and vessel two to three through the waste pipe of each vessel.

The human gut model media from MacFarlane *et al.* 1998 (8) was altered to mirror the ADF and fibre content seen in compositional analysis of gastrointestinal

content (Figure S1). To represent the normal diet of a horse the percentage of starch and fibre were increased and the percentage of protein decreased. The composition of this media in distilled water was: cellulose (15 g $litre^{-1}$), yeast extract (5 g $litre^{-1}$), NaCl (5 g $litre^{-1}$), KCl (5 g $litre^{-1}$), mucin (4 g $litre^{-1}$), raffinose (3.5 g $litre^{-1}$), starch (2 g $litre^{-1}$), peptone water (1.5 g $litre^{-1}$), tryptone (1.5 g $litre^{-1}$), $NaHCO_3$ (1.5 g $litre^{-1}$), $MgSO_4(.7H_2O)$ (1.25 g $litre^{-1}$), arabinogalactan (1 g $litre^{-1}$), xylan (0.835 g $litre^{-1}$), cysteine.HCl (0.8 g $litre^{-1}$), KH_2PO_4 (0.5 g $litre^{-1}$), K_2HPO_4 (0.5 g $litre^{-1}$), bile salts (0.4 g $litre^{-1}$), $CaCl_2$.6H2O (0.15 g $litre^{-1}$), hemin (0.05 g $litre^{-1}$), $FeSO_4.H_2O$ (0.005 g $litre^{-1}$), tween 80 (0.5 ml $litre^{-1}$) and vitamin K (0.01 ml $litre^{-1}$).

To inoculate the gut model a sample of feces was collected from the rectum *post-mortem* or immediately after defecation for non-invasive sample acquisition and placed in a sealed jar, with an anaerobic atmosphere generating sachet (Oxoid AnaeroGen 2.5 L Sachet; Thermo Fisher) for transportation for a maximum of two hours. A 20 % fecal slurry (1 x PBS) was made in a strainer stomacher bag (Seward), by homogenizing fecal balls manually within an anaerobic cabinet (Whitley A35 anaerobic workstation). 100 ml, 133 ml and 67 ml of the fecal slurry was decanted and added respectively to each vessel (sample taken immediately after inoculation were designated T-1). After the fecal slurry was added the model was allowed to equilibrate as a batch culture for 24 hours. The media pump was started after 24 hours, designated T0. The short period of batch culture allowed the fecal bacteria to acclimatise to their new environment and reduce the washing-out of bacteria when the flow of media was started.

A sample of 5 ml was taken through the sample port of each vessel at T-1, T0 and every subsequent full turnover of media through the model (every three days). Two aliquots of 1 ml were centrifuged at 11,337 *g*, the supernatant stored at -20 °C

and pellets stored at -80 °C. For preparation of FISH slides, an aliquot of 375 µl from each sample was added to 1125 µl 4 % paraformaldehyde (PFA) and incubated at 5 °C for four hours. PFA was washed off with 1 x PBS, the pellet re-suspended in 150 µl 1 x PBS and 150 µl ethanol and stored at -20 °C until required. Processing of all samples was carried out in an anaerobic cabinet (Whitley A35 anaerobic workstation).

Concordance and repeatability studies.

The concordance study used three gut models (M1, M2 and M3) inoculated with feces from three different horses to assess the similarity between the gastrointestinal samples and the stabilised fermentation model of the same horse. Modelling three different individuals allowed assessment of whether the model could capture the inter-individual variation seen *in vivo*. Samples of gastrointestinal content were taken *post mortem* from the cecum, right ventral colon, left ventral colon and feces from the rectum of three horses (Horses 4, 5 and 6 in Table S1). These samples were transported on dry ice and then stored at -80 °C until defrosted for analyses. A further 100 g of feces were taken from of each horse and stored in an anaerobic jar (Oxoid) with an anaerobic gas generating sachet until it was used to make a 20 % fecal slurry used to inoculate all vessels. Each gut model was sampled every turnover until steady state was reached (T5, when SCFA production stabilised).

Subsequent to this, two gut models (M4 and M5) were run at the same time with feces from the same horse, for the repeatability study. This aimed to assess the reproducibility of the metabolic and bacterial signatures between different gut models. Freshly voided feces were collected from a healthy horse (Horse 7 in Table S1) and transported in a sealed container with an anaerobic gas generating sachet to use for

fecal slurry inoculum. These models were run until turnover seven (T7) to assess stability of the model after steady state (T5) was reached and samples were taken and processed as previously described. An overview of the inoculation and sampling of the concordance and repeatability studies can be seen in Figure 8.

SCFA/BCFA analysis by Gas Chromatography.

Supernatants from all gut models at all time points underwent gas chromatography to analyse the levels of acetate, propionate, butyrate, isobutyrate and valerate. These samples were analysed using an acidification method adapted from Zhao *et al.* 2006 (32). Samples were filtered through 0.2 mm PVDF filer and acidified to pH 2 using sulfuric acid. An internal standard of 2-ethyl butyrate was added to at 5 mM in each sample. A fused-silica capillary column with a free fatty acid phase (DB-FFAP) was used; with helium as the carrier gas, flow rate of 14.4 ml/min. The initial oven temperature was 100 °C, which was held for 2 minutes before being raised to 180 °C at 8 °C per minute and held for a further 2 minutes, then increased to 200 °C at 20 °C per minute, and held at 200 °C for 5 minute. A range of standards were used at concentrations from 0.5mM to 100mM, these contained acetate, propionate, butyrate, iso-butyrate, valerate and isovalerate. Analysis of peak areas was done using ChemStation B.03.01 software (Aligent Technologies).

Human gut models are often run for eight turnovers (24 days) before they are deemed to reach steady state (assessed by stabilization of SCFA levels) and a drug or feed intervention added (e.g. Grimaldi et al. 2017). We chose to use SCFA production as a marker for model stability, plateauing of production indicating achievement of steady state (Fig 1). Accordingly, community profiling models were run

until T5 (15 days after media flow was started) as SCFA production was deemed to be stable at this time point; the repeatability study was run for a further two turnovers after this point to further assess the stability of the model. Gut models maintained for longer time-periods (data not reported here) have confirmed stability of SCFA production until T7 (1 days after media flow was started).

Kruskal-Wallis tests were performed to assess the stability of the SCFA produced by the vessels of the concordance study gut models from T3 to T5 to assess if steady state had been reached. Kriskall-Wallis tests were also used to assess the stability of SCFA production in the repeatability study gut models from T3 to T5, from T5 to T7 and whether there were any differences in SFCA production between the two models created with the same fecal inoculum (repeatability study).

^{1}H NMR spectroscopy.

Sample supernatants taken from the model were prepared for ^{1}H NMR analysis by adding 200 µl of phosphate buffer (pH 7.4; 100 % D_2O) containing 1 mM of the internal standard 3-trimethylsilyl-1-[2,2,3,3-^{2}H4] propionate (TSP) to 400 µl of each sample and transferred into 5 mm NMR tubes. Spectroscopic analysis of all samples was carried out on a 700 MHz Bruker NMR spectrometer equipped with a cryo-probe. Standard one-dimensional ^{1}H NMR spectra were acquired for all samples with water peak suppression using a standard pulse sequence. For each sample, 8 dummy scans were followed by 256 scans and collected in 64K data point. Chemical shifts in the fecal spectra were referenced to the TSP singlet at δ 0.0. Scans collected 64K data points. A spectral width of 20 ppm and an acquisition time per scan of 3.12 seconds were used.

529 ^{1}H NMR spectra were analysed in the Matlab environment (R2014a;
530 Mathworks) with in-house scripts. Spectra were initially aligned and normalized
531 (probabilistic quotient method) before multivariate models were built to compare the
532 metabolic profiles of gastrointestinal content to sample supernatants from the gut
533 model. Initial unsupervised PCA models were constructed to identify metabolites that
534 explain the largest sources of variation within the dataset. OPLS-DA models were
535 constructed for pair-wise comparisons of gastrointestinal content and gut model
536 supernatants. The predictive ability (Q^2Y) of the OPLS-DA models were calculated
537 using seven-fold cross validation. Metabolites were assigned to peaks using the
538 database of equine metabolites found in Escalona e*t al.* (33).

539

540 **FISH analysis for total bacteria.**

541 FISH was used alongside 16S gene sequencing in order to enumerate total bacterial
542 numbers. It provides a validated approach for counting microbial numbers using
543 probes targeting specific bacterial taxa. FISH analyses were carried out by a trained
544 operator; quality assurance was provided by duplicate counting by second operator
545 which gave rise to comparable results (greater than 95% confidence of similarity).
546 Total bacterial count per ml was calculated by utilising Eubmix FISH probe. This is a
547 mixture of three EUB338 probes (I, II and III, Sigma Aldrich) with the dye Cy3 tagged
548 at the 5' end. Hybridisation was performed as described in Daims *et al.* 1999 (34). All
549 samples prepared for FISH were diluted to 1 in 100 (in PBS:SDS) and 20 µl was added
550 to a well of Teflon- and poly L-lysine-coated 6-well slide (Tekdon Inc.). Slides were
551 dried on a plate incubator for 15 minutes at 46 - 50 °C, dehydrated in 30 %, 80 % and
552 96 % ethanol for 3 minutes each and then dried for 2 minutes. Hybridisation mixture

(0.9 M NaCl, 0.02 M Tris/HCl (pH 8), formamamide, 10 % sodium dodecyl sulphate and 4.55 ng ml^{-1} probe) was added to each well, slides placed on a sealed tray and put in a hybridisation oven for 4 hours at 46 °C. Once hybridisation was complete slides were placed into wash buffer (0.9 M NaCl, 0.02 M Tris/HCl (pH 8) 0.005 M EDTA solution (pH 8), H_2O) for 10 - 15 minutes at 48 °C. After washing slides were dipped into cold water for 2 - 3 seconds and dried using compressed air. Once dry, antifade solution (Dabco) was added to each well and a cover slip applied. Fixed bacteria were visualised using fluorescence microscopy (Nikon Eclipse Ni-U microscope), the number of bacteria counted in 15 random fields of view per well and a mean calculated. To calculate the number of bacteria per ml the following equation was used:

$$Bacteria\ per\ ml = 0.8 \times mean\ bacteria \times 6788.42 \times 50 \times 100$$

Preparation and analysis of bacterial DNA sequencing.

DNA was extracted from frozen gastrointestinal samples and pellets from the gut model using PSP® spin stool DNA plus kit (Invisorb) using the manufacturer's protocol. Extracted DNA was re-suspended in 100 µl of elution buffer. Further preparation and DNA sequencing was carried out by the Centre of Genomic Research, Liverpool. The extracted DNA was amplified using PCR of the V4 region of the 16S rRNA gene using the primers F515 (GTGCCAGCMGCCGCGGTAA) and 806R (GGACTACHVGGGTWTCTAAT) (35), with reverse primers containing individual Golay barcodes. For a final volume of 20 µl to be subjected to V4 PCR 1 µl of the extracted DNA from each sample was added to: 7 µl of molecular water, 10 µl NEBNext High Fidelity Master Mix (New England Biolabs), 1 µl of forward primer (3 µM) and 1 µl of an individually barcoded reverse primer (3 µM). This mixture was made

for each individual sample, which then underwent the following PCR conditions: 30 seconds at 98 °C and 25 cycles of: 10 seconds at 98 °C, 30 seconds at 55 °C and 30 seconds at 72 °C. This was followed by a final period of 5 minutes at 72 °C and then kept at 4 °C until processed. PCR products were evaluated by electrophoresis in 2 % agarose gel stained with midori green. Successfully amplified DNA was cleaned using AMPure XP beads (Agencourt) and quantified using the Quant-iT PicoGreen dsDNA assay (Life Tech). PCR products were pooled at an equimolar ratio and size selected using a 2 % agarose gel cassette run in a Pippin Prep (Sage Science machine) where fragments at 254 bp were eluted and kept for sequencing. Sequencing was carried out on the MiSeq Illumina platform.

Analysis of sequencing data was carried out on a remote linux server provided by the University of Surrey with QIIME2 (36) installed. Zipped read files were uploaded to the linux server and converted into a QIIME artefact (qiime tools import*)* and a summary was generated (qiime demux summarize*)*. Quality control was carried out using DADA2 (37) and the ends of reads were trimmed at position 15 and 220 of the reads (qiime dada2 denoise-single*)*. A rooted phylogenetic was generated for diversity analyses (qiime alignment mafft, qiime alignment mask, qiime phylogeny fasttree and qiime phylogeny midpoint-root). Diversity core metrics were generated at a sampling depth of 47,000 reads (qiime diversity core-metrics-phylogenetic) and boxplots displaying alpha diversity (measure as observed OTUs) were generated (qiime diversity alpha-group-significance). The reference database greengenes (38) was downloaded and the taxanomic classifiers were trained on this specific sample preparation and sequencing parameters (qiime feature-classifier classify-sklearn). Taxa summary barplots were generated (qiime taxa barplot).

Mann-Whitney U tests were used to assess whether read numbers for identified bacterial phyla were statistically different when comparing gastrointestinal content to the gut model and time points of the model. Regression models were built with the mean SCFA/BCFA concentration and bacterial phyla count for the each vessel of the concordance study at T-1, T0 and T5. Correlations identified by the models were deemed to be significant when $R^2 = 0.5$ and $p < 0.05$. Venn diagrams were generated to visualise how many identifiable and uniquely named OTUs were shared between the vessels of the three biological replicate gut models and their corresponding gastrointestinal compartments.

Raw sequencing reads analysed in this manuscript have been submitted to the European Nucleotide Archive and can be found in project ERP107630.

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REFERENCES

1. Dougal K, Harris PA, Edwards A, Pachebat JA, Blackmore TM, Worgan HJ, et al. A comparison of the microbiome and the metabolome of different regions of the equine hindgut. FEMS Microbiol Ecol. 2012;82(3):642–52.

2. Costa MC, Silva G, Ramos R V., Staempfli HR, Arroyo LG, Kim P, et al.

625 Characterization and comparison of the bacterial microbiota in different
626 gastrointestinal tract compartments in horses. Vet J [Internet]. 2015;205(1):74–
627 80. Available from:
628 http://dx.doi.org.idpproxy.reading.ac.uk/10.1016/j.tvjl.2015.03.018

629 3. Proudman CJ, Hunter JO, Darby AC, Escalona EE, Batty C, Turner C.
630 Characterisation of the faecal metabolome and microbiome of Thoroughbred
631 racehorses. Equine Vet J. 2015;47(5):580–6.

632 4. Costa MC, Stämpfli HR, Allen-Vercoe E, Weese JS. Development of the faecal
633 microbiota in foals. Equine Vet J. 2016;48(6):681–8.

634 5. Ericsson AC, Johnson PJ, Lopes MA, Perry SC, Lanter R. A Microbiological
635 Map of the Healthy Equine Gastrointestinal Tract. PLoS One. 2016;11(11):1–
636 17.

637 6. Costa MC, Arroyo LG, Allen-Vercoe E, Stämpfli HR, Kim PT, Sturgeon A, et al.
638 Comparison of the fecal microbiota of healthy horses and horses with colitis by
639 high throughput sequencing of the V3-V5 region of the 16s rRNA gene. PLoS
640 One. 2012;7(7):e41484.

641 7. Leng J, Proudman C, Darby A, Blow F, Townsend N, Miller A, et al.
642 Exploration of the Fecal Microbiota and Biomarker Discovery in Equine Grass
643 Sickness. J Proteome Res. 2018;17(3):1120–8.

644 8. Macfarlane GT, Macfarlane S, Gibson GR. Validation of a three-stage
645 compound continuous culture system for investigating the effect of retention
646 time on the ecology and metabolism of bacteria in the human colon. Microb
647 Ecol. 1998;35(2):180–7.

9. Walton GE, Heuvel EGHM Van Den, Kosters MHW, Rastall RA, Tuohy KM, Gibson GR. A randomised crossover study investigating the effects of galacto-oligosaccharides on the faecal microbiota in men and women over 50 years of age. Br J Nutr. 2012;107:1466–75.

10. Grimaldi R, Cela D, Swann JR, Vulevic J, Gibson GR, Tzortzis G, et al. In vitro fermentation of B-GOS : Impact on faecal bacterial populations and metabolic activity in autistic and non-autistic ... In vitro fermentation of B-GOS : impact on faecal and non-autistic children. FEMS Micro Ecolo. 2017;93(2):1–10.

11. Grimaldi R, Gibson GR, Vulevic J, Giallourou N, Castro-mejía JL, Hansen LH, et al. A prebiotic intervention study in children with autism spectrum disorders (ASDs). Microbiome. 2018;6(133):1–13.

12. Lowman RS, Theodorou MK, Hyslop JJ, Dhanoa MS, Cuddeford D. Evulation of an in vitro batch culture technique for estimating the in vivo digestibility and digestible energy content of equine feeds using equine faeces as the source of microbial inoculum. Anim Feed Sci Technol. 1999;80(1):11–27.

13. Desrousseaux G, Santos AS, Pellikaan WF, Van der Poel AFB, Cone JW, Guedes CM V, et al. Effect of collection time on the fermentative activity of microbes in equine faeces. Anim Feed Sci Technol [Internet]. 2012;178(3–4):183–9. Available from: http://dx.doi.org/10.1016/j.anifeedsci.2012.09.016

14. Biddle AS, Black SJ, Blanchard JL. An In Vitro Model of the Horse Gut Microbiome Enables Identification of Lactate-Utilizing Bacteria That Differentially Respond to Starch Induction. PLoS One. 2013;8(10):1–13.

15. Andoh A, Tsujikawa T, Sasaki M, Mitsuyama K, Suzuki Y, Matsui T, et al. Faecal microbiota profile of Crohn’s disease determined by terminal restriction

672 fragment length polymorphism analysis. Aliment Pharmacol Ther.
673 2009;29(1):75–82.

674 16. Moore-Colyer M, O'Gorman DM, Wakefield K. An In Vitro Investigation into the
675 Effects of a Marine-Derived, Multimineral Supplement in Simulated Equine
676 Stomach and Hindgut Environments. J Equine Vet Sci [Internet].
677 2014;34(3):391–7. Available from:
678 http://linkinghub.elsevier.com/retrieve/pii/S0737080613004735

679 17. Murray JAMD, Bice RKT, Moore-Colyer MJS. The effect of particle size on the
680 in vitro fermentation of different ratios of high-temperature dried lucerne and
681 sugar beet pulp incubated with equine faecal inocula. Anim Feed Sci Technol
682 [Internet]. 2010;162(1–2):47–57. Available from:
683 http://dx.doi.org/10.1016/j.anifeedsci.2010.09.001

684 18. Murray JAMD, McMullin P, Handel I, Hastie PM. The effect of freezing on the
685 fermentative activity of equine faecal inocula for use in an in vitro gas
686 production technique. Anim Feed Sci Technol [Internet]. 2012;178(3–4):175–
687 82. Available from: http://dx.doi.org/10.1016/j.anifeedsci.2012.09.013

688 19. Murray JMD, McMullin P, Handel I, Hastie PM. Comparison of intestinal
689 contents from different regions of the equine gastrointestinal tract as inocula
690 for use in an in vitro gas production technique. Anim Feed Sci Technol
691 [Internet]. 2014;187:98–103. Available from:
692 http://dx.doi.org/10.1016/j.anifeedsci.2013.10.005

693 20. Murray JAMD, Scott B, Hastie PM. Fermentative capacity of equine faecal
694 inocula obtained from clinically normal horses and those predisposed to
695 laminitis. Anim Feed Sci Technol. 2009;151(3–4):306–11.

21. Abdouli H, Attia S Ben. Evaluation of a two-stage in vitro technique for estimating digestibility of equine feeds using horse faeces as the source of microbial inoculum. Anim Feed Sci Technol. 2007;132(1–2):155–62.

22. Elghandour MMY, Vázquez Chagoyán JC, Salem AZM, Kholif AE, Martínez Castaneda JS, Camacho LM, et al. In Vitro fermentative capacity of equine fecal inocula of 9 fibrous forages in the presence of different doses of Saccharomyces cerevisiae. J Equine Vet Sci [Internet]. 2014;34(5):619–25. Available from: http://dx.doi.org/10.1016/j.jevs.2013.11.013

23. Hobden MR, Martin-morales A, Guérin-deremaux L, Wils D, Costabile A, Walton GE, et al. In Vitro Fermentation of NUTRIOSE ® FB06 , a Wheat Dextrin Soluble Fibre , in a Continuous Culture Human Colonic Model System. PLoS One. 2013;8(10):1–7.

24. Costabile A, Walton GE, Tzortzis G, Vulevic J, Charalampopoulos D, Gibson GR. Effects of Orange Juice Formulation on Prebiotic Functionality Using an In Vitro Colonic Model System. PLoS One. 2015;10(3):1–12.

25. Pérez-López E, Cela D, Costabile A, Mateos-Aparicio I, Rupérez P. In vitro fermentability and prebiotic potential of soyabean Okara by human faecal microbiota. Br J Nutr. 2016;116(6):1116–24.

26. Liu Y, Gibson GR, Walton GE. A three-stage continuous culture approach to study the impact of probiotics , prebiotics and fat intake on faecal microbiota relevant to an over 60 s population. J Funct Foods [Internet]. 2017;32:238–47. Available from: http://dx.doi.org/10.1016/j.jff.2017.02.035

27. Gharbia SE, Shah HN. Pathways of glutamate catabolism among Fusobacterium species. J Gen Microbiol. 1991;137(5):1201–6.

720 28. Rogers AH, Chen J, Zilm PS, Gully NJ. The Behaviour of Fusobacterium
721 nucleatum Chemostat-grown in Glucose- and Amino Acid-based Chemically
722 Defined Media. Anaerobe. 1998;4(2):111–6.

723 29. Macrae JC, Armstrong DG. Enzyme Method for Determination of Alpha-
724 Lunked Glucose Polymers in Biological Materials. J Sci Food Agric.
725 1968;19(10):578.

726 30. Fuller KW. Automated Determination of Sugars. Tech Symp. 1966;11(57).

727 31. Macfarlane GT, Englyst HN. Starch utilization by the human large intestinal
728 microflora. J Appl Bacteriol. 1986;60(3):195–201.

729 32. Zhao G, Nyman M, Jönsson JÅ. Rapid determination of short-chain fatty acids
730 in colonic contents and faeces of humans and rats by acidified water-extraction
731 and direct-injection gas chromatography. Biomed Chromatogr.
732 2006;20(8):674–82.

733 33. Escalona EE, Leng J, Dona AC, Merrifield CA, Holmes E, Proudman CJ, et al.
734 Dominant components of the Thoroughbred metabolome characterised by 1H-
735 nuclear magnetic resonance spectroscopy: A metabolite atlas of common
736 biofluids. Equine Vet J. 2015;47(6):721–30.

737 34. Daims H, Bruhl A, Amann R, Schleifer K, Wagner M. The Domain-specific
738 Probe EUB338 is Insufficient for the Detection of all Bacteria: Development
739 and Evaluation of a more Comprehensive Probe Set. Syst Appl Microbiol.
740 1999;444(22):434–44.

741 35. Caporaso JG, Lauber CL, Walters WA, Berg-lyons D, Lozupone CA,
742 Turnbaugh PJ, et al. Global patterns of 16S rRNA diversity at a depth of

743 millions of sequences per sample. Proc Natl Acad Sci U S A.
744 2011;108(1):4516–22.

745 36. Bolyen E, Rideout J, Dillon M, Bokulich N, Abnet C, Al-Ghalith G, et al. QIIME
746 2: Reproducible, interactive, scalable, and extensible microbiome data
747 science. PeerJ Prepr. 2018;6:e27295v2.

748 37. Callahan BJ, Mcmurdie PJ, Rosen MJ, Han AW, Johnson AJA, Holmes SP.
749 DADA2: High resolution sample inference from Illumina amplicon data. Nat
750 Methods. 2016;13(7):581–3.

751 38. Mcdonald D, Price MN, Goodrich J, Nawrocki EP, Desantis TZ, Probst A, et al.
752 An improved Greengenes taxonomy with explicit ranks for ecological and
753 evolutionary analyses of bacteria and archaea. ISME J [Internet].
754 2012;6(3):610–8. Available from: http://dx.doi.org/10.1038/ismej.2011.139

755

756 **Figures**

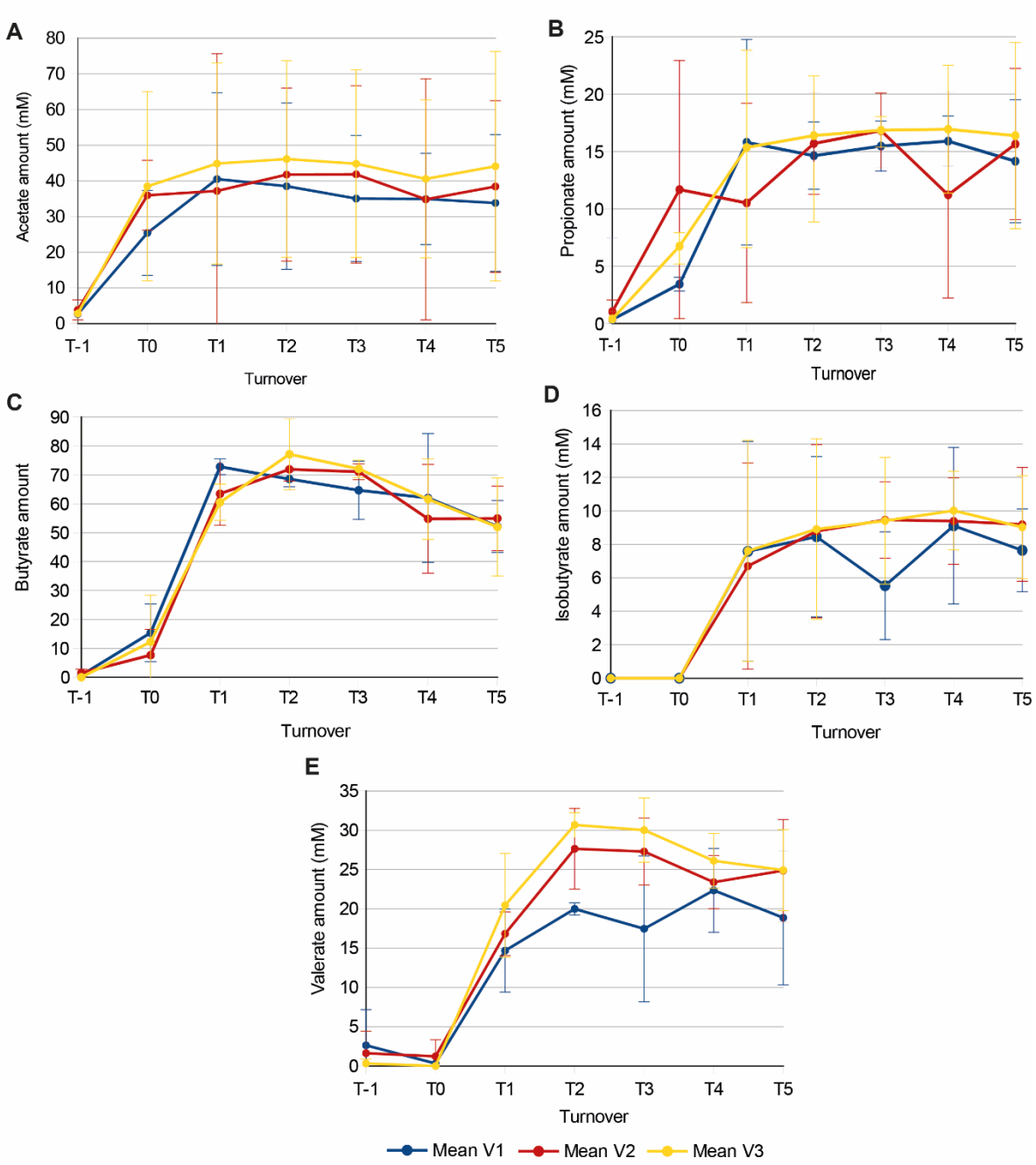
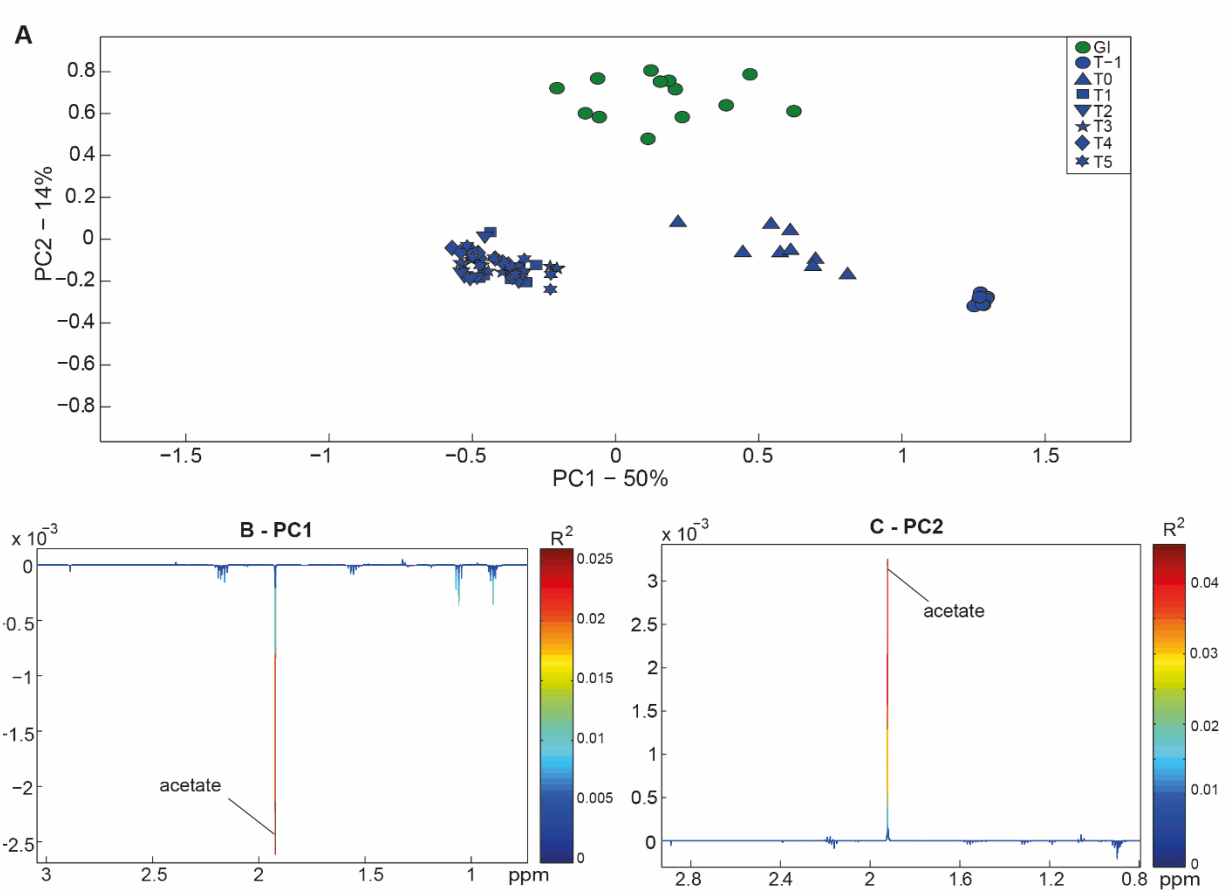


Figure 1: Demonstration of biological functionality of the gut models in the concordance study. SCFA/BCFA production of the three separate gut models inoculated with feces from three different horses during the concordance study (shown as the mean and standard deviation of the three vessels of the model). The SFCA/BCFAs measured were: A) acetate, B) propionate, C) butyrate, D) isobutyrate and E) valerate. T, turnover; V, vessel.



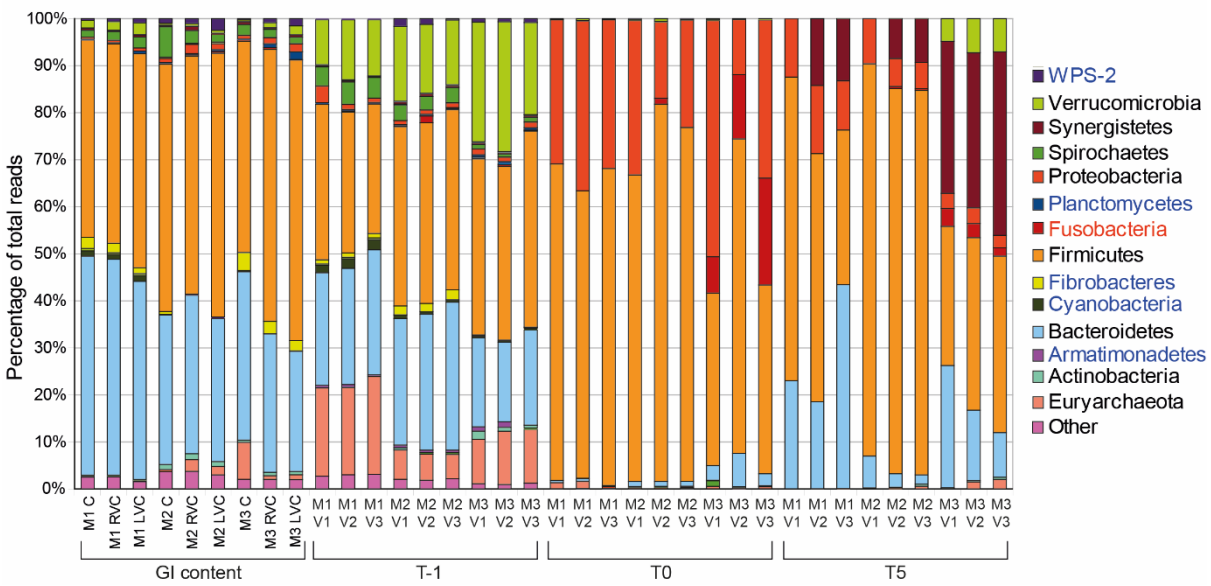
764 **Figure 2:** Biochemical variation (measured by ^{1}H NMR spectroscopy) between the gut
765 contents of donor horses and *in-vitro* gut model supernatant from the concordance
766 study. A) PCA scores plot for PC1 and PC2 of the model (R^2 = 0.64). B)The loading
767 plot for PC1, describing 50 % of the total variance associated with higher concentration
768 of acetate in model supernatant at time poinrs T1 – T5 and C) the loading plot for PC2
769 describing 14 % of the total variance, associated with higher acetate concentration in
770 gut contents. PC, principle component; T, turnover.

771

772

773

774 **Figure 3:** Relative abundance of the bacterial phyla (assigned by 16S bacterial gene
775 sequencing) identified in the gastrointestinal content from donor horses and the three



776 gut models from the concordance study. The key indicates whether bacterial phyla
777 are: identified in both gastrointestinal and gut model samples at T5 (black), only in
778 gastrointestinal samples (blue) or only in gut model samples (red).

779 C, cecum; RVC, right ventral colon; LVC, left ventral colon; V, vessel; T, turnover; M,
780 model.

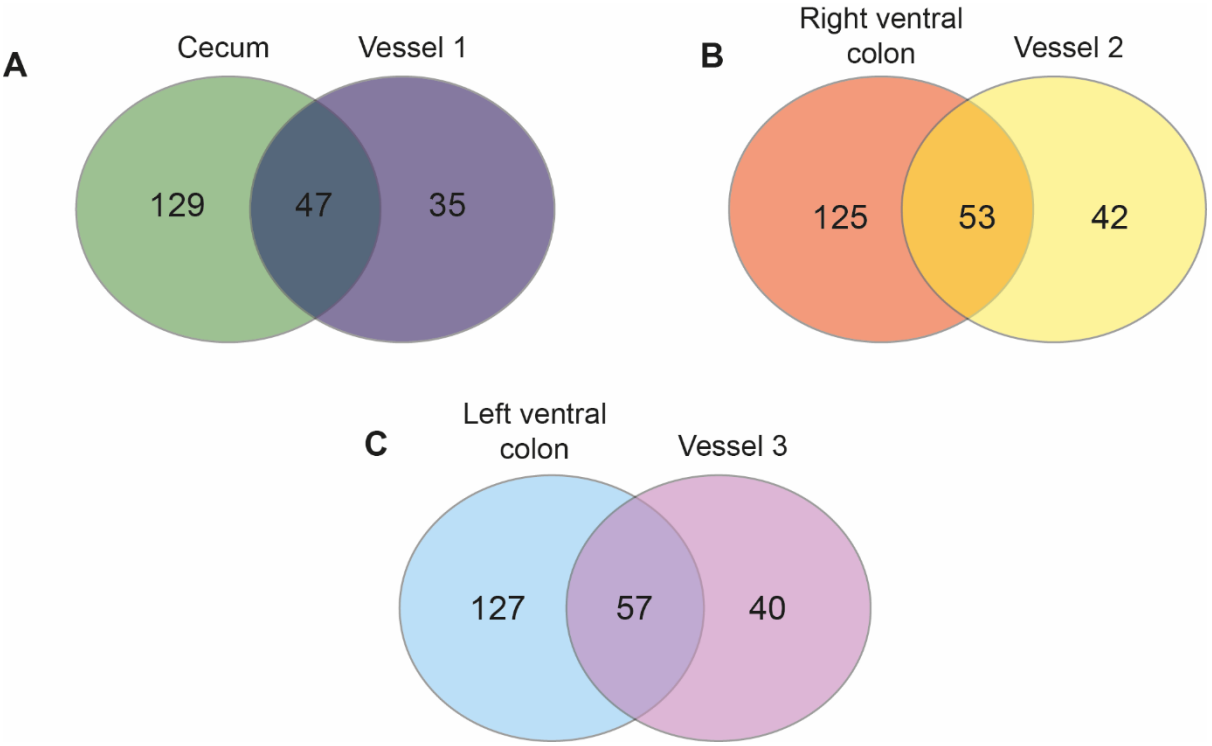


Figure 4: Venn diagram showing the total number of distinct OTUs that are unique and shared between samples taken from the areas of the equine large intestine and their corresponding vessels from the gut models. These models were from the concordance study used to capture individual variation.

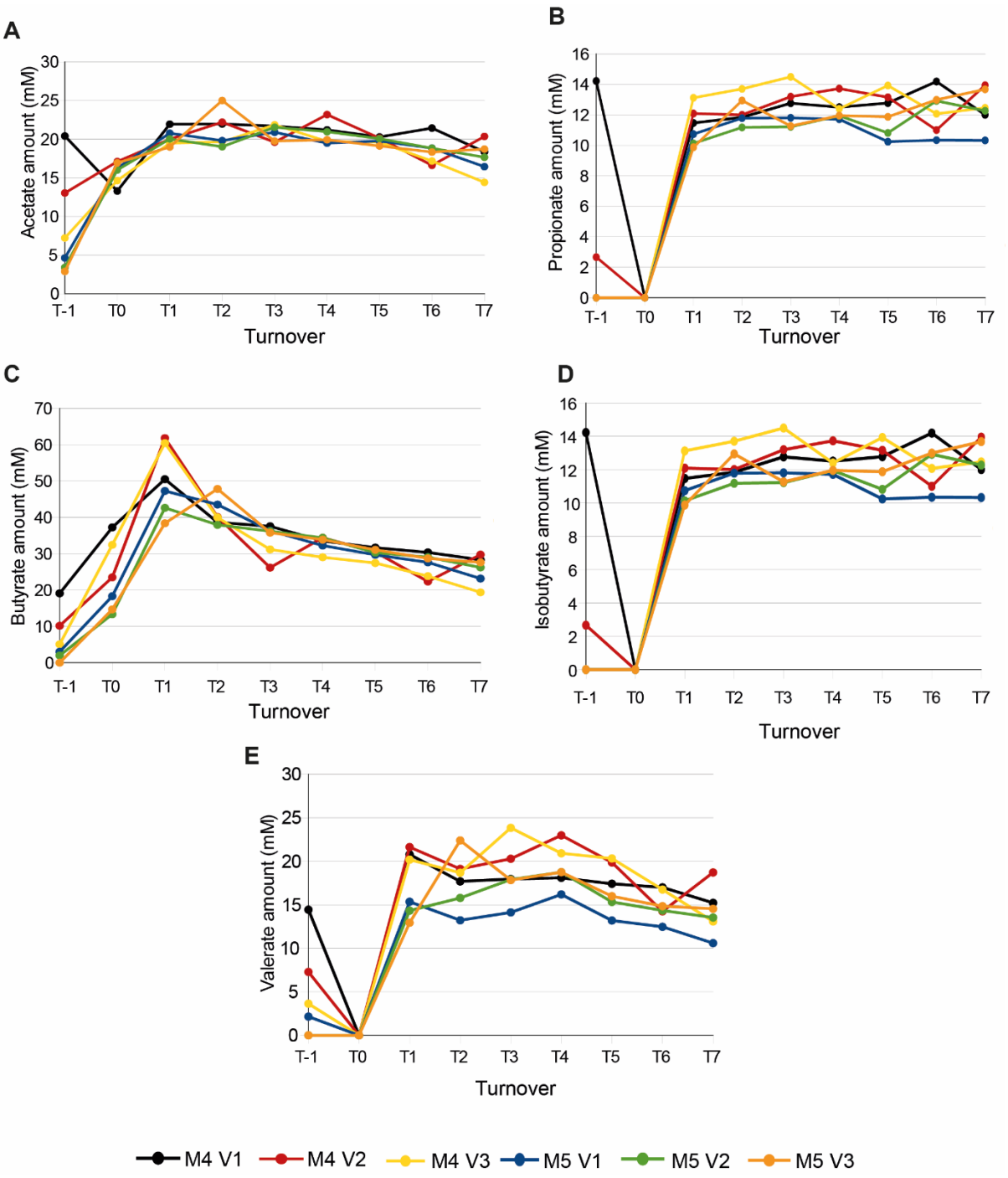


Figure 5: Demonstration of repeatability of biological function between models. SCFA/BCFA production (measured by gas chromatography) in each vessel of the models was inoculated with the same feces (repeatability study). Each line represents the level of a SCFA/BCFA in a single vessel of one of the two gut models of the repeatability study. The SFCA/BCFAs measured were: A) acetate, B) propionate, C) butyrate, D) isobutyrate and E) valerate. T, turnover; R, technical replicate; V, vessel; M, model.

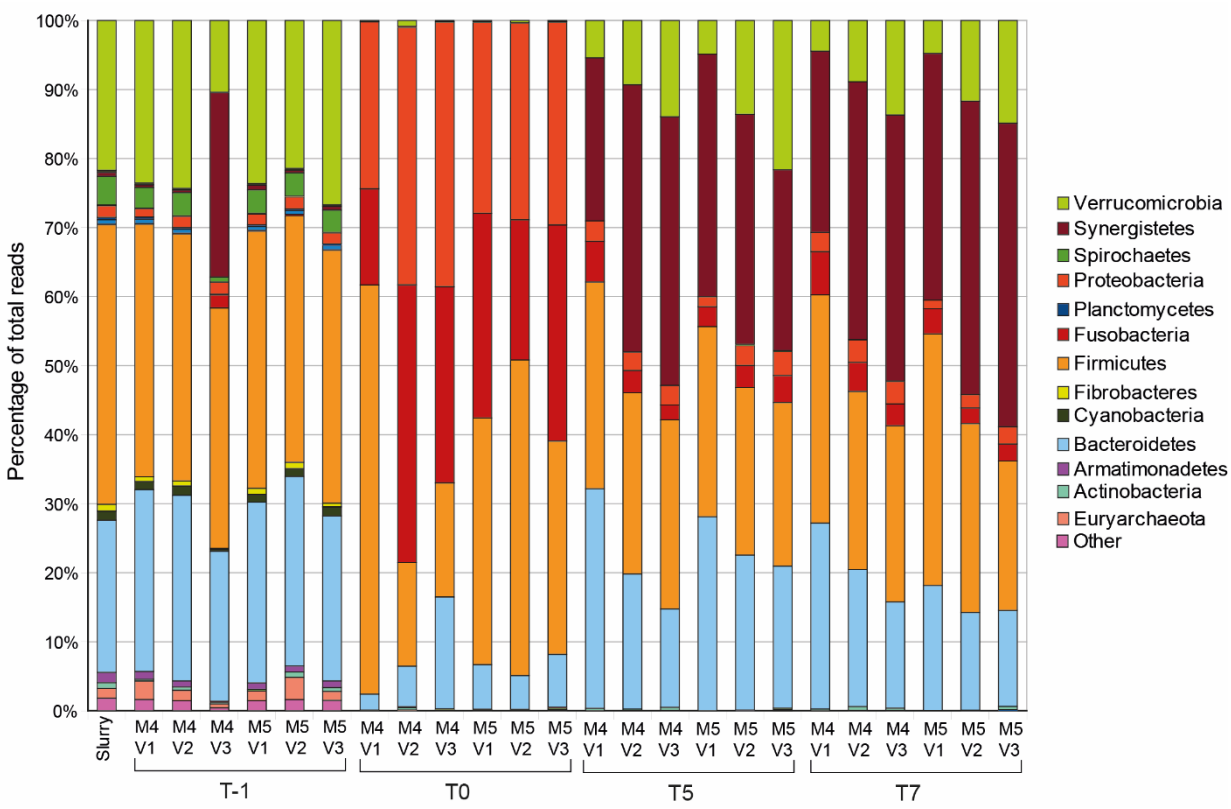


Figure 6: Demonstration of the repeatability of model bacterial communities. Taxonomic assignments (phyla from 16S bacterial gene sequencing) from the repeatability study; two models inoculated with feces from the same horse. M, model; V, vessel; T, turnover.

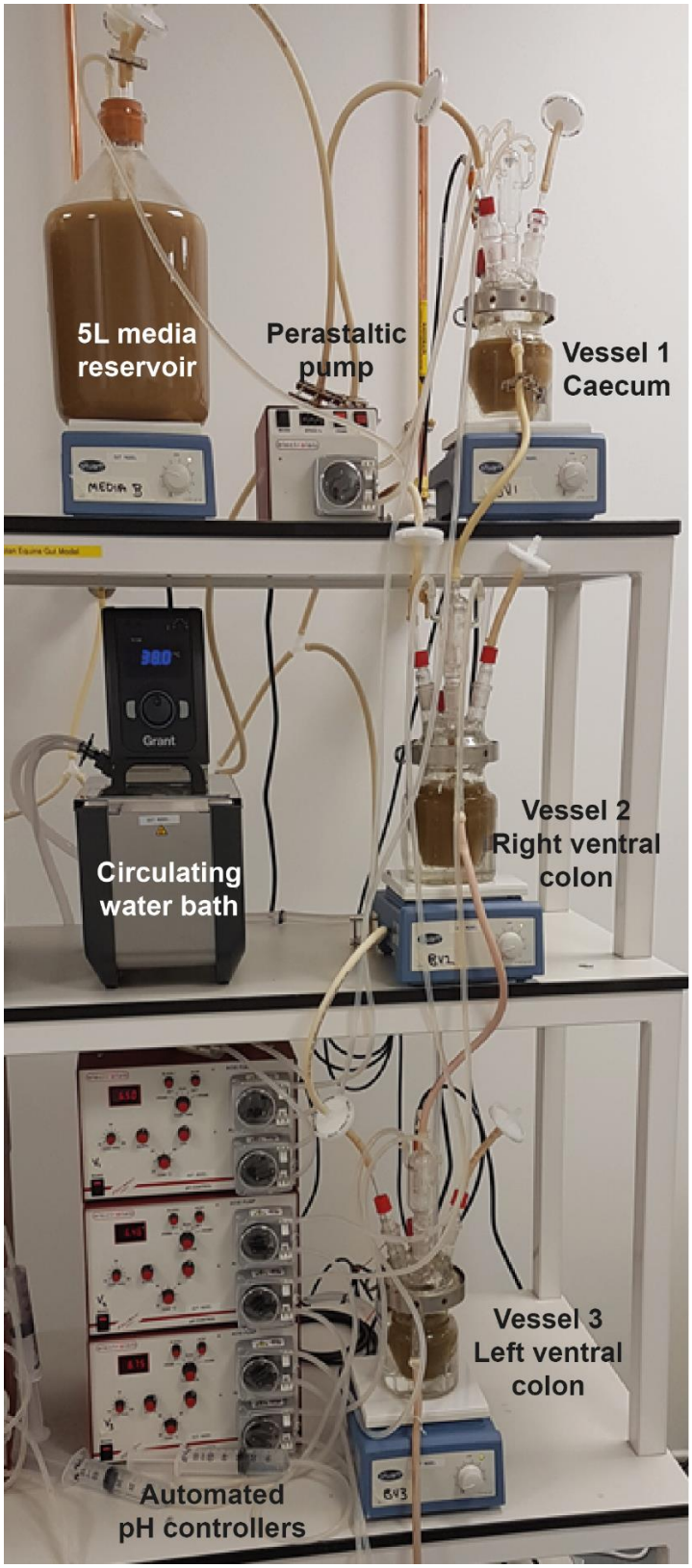

5L media reservoir
Perastaltic pump
Vessel 1 Caecum
Circulating water bath
Vessel 2 Right ventral colon
Vessel 3 Left ventral colon
Automated pH controllers

Figure 7: Three stage, *in vitro*, fermentation model designed to replicate the microbiota of the equine cecum, right ventral colon and left ventral colon (modified from MacFarlane *et al.* 1998).

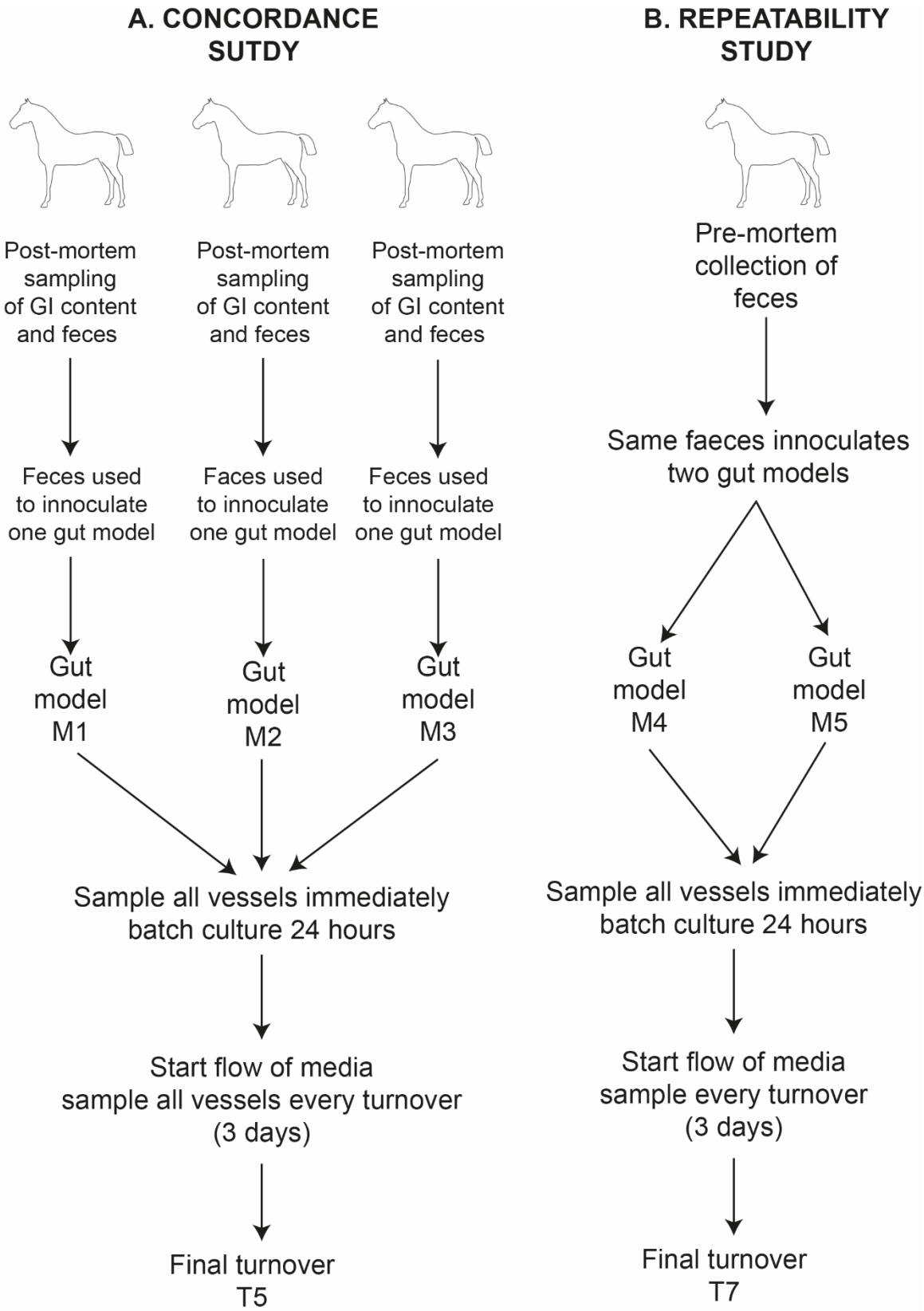


Figure 8: Schematic showing the sampling scheme and experimental design for A) concordance study to demonstrate capture of inter-individual variation and B) repeatability study to demonstrate consistency of models inoculated with the same feces.

Supplementary Figures

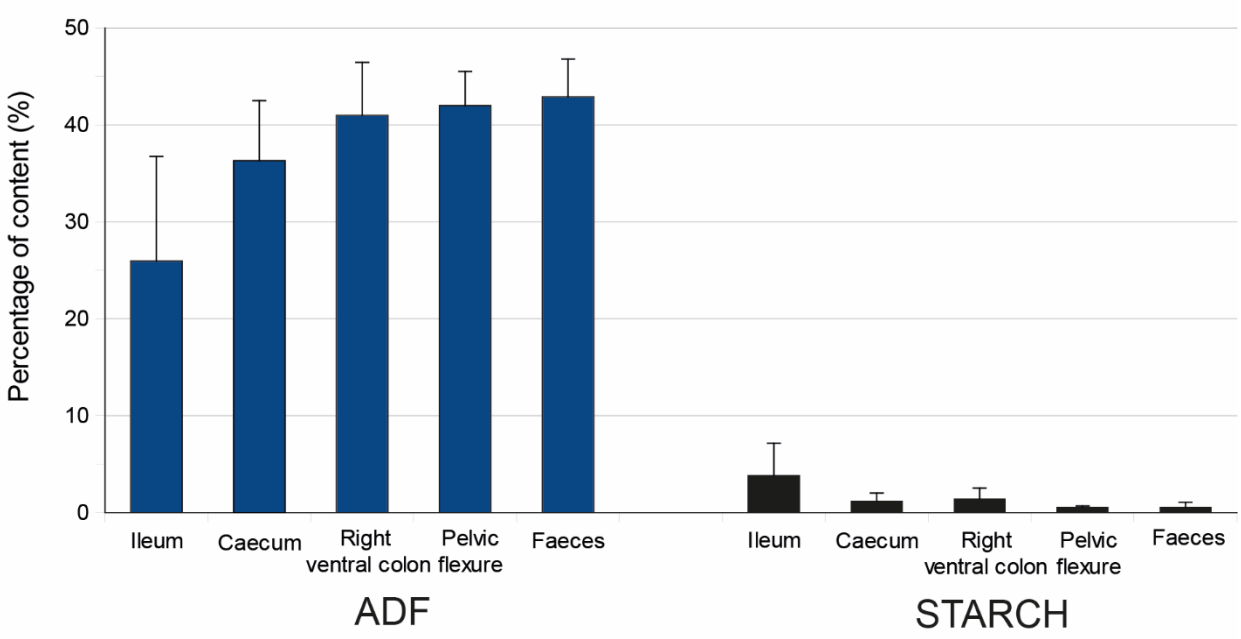
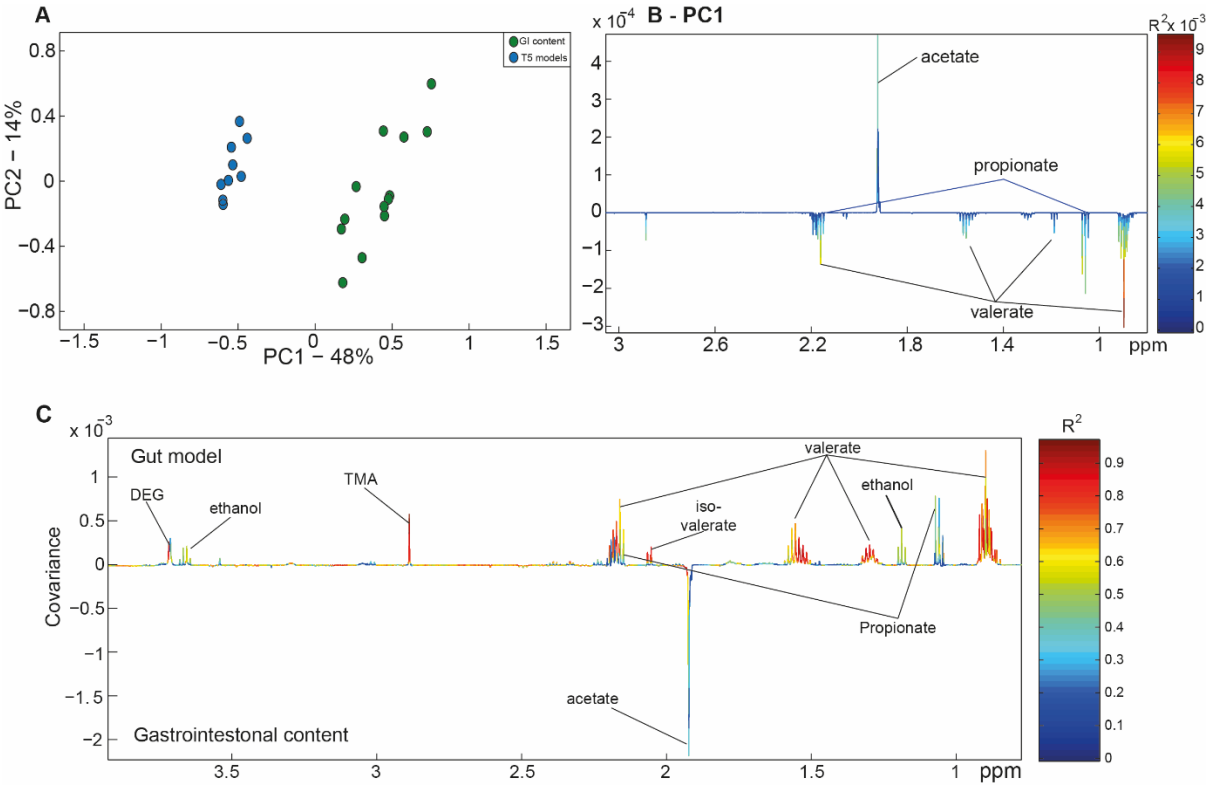
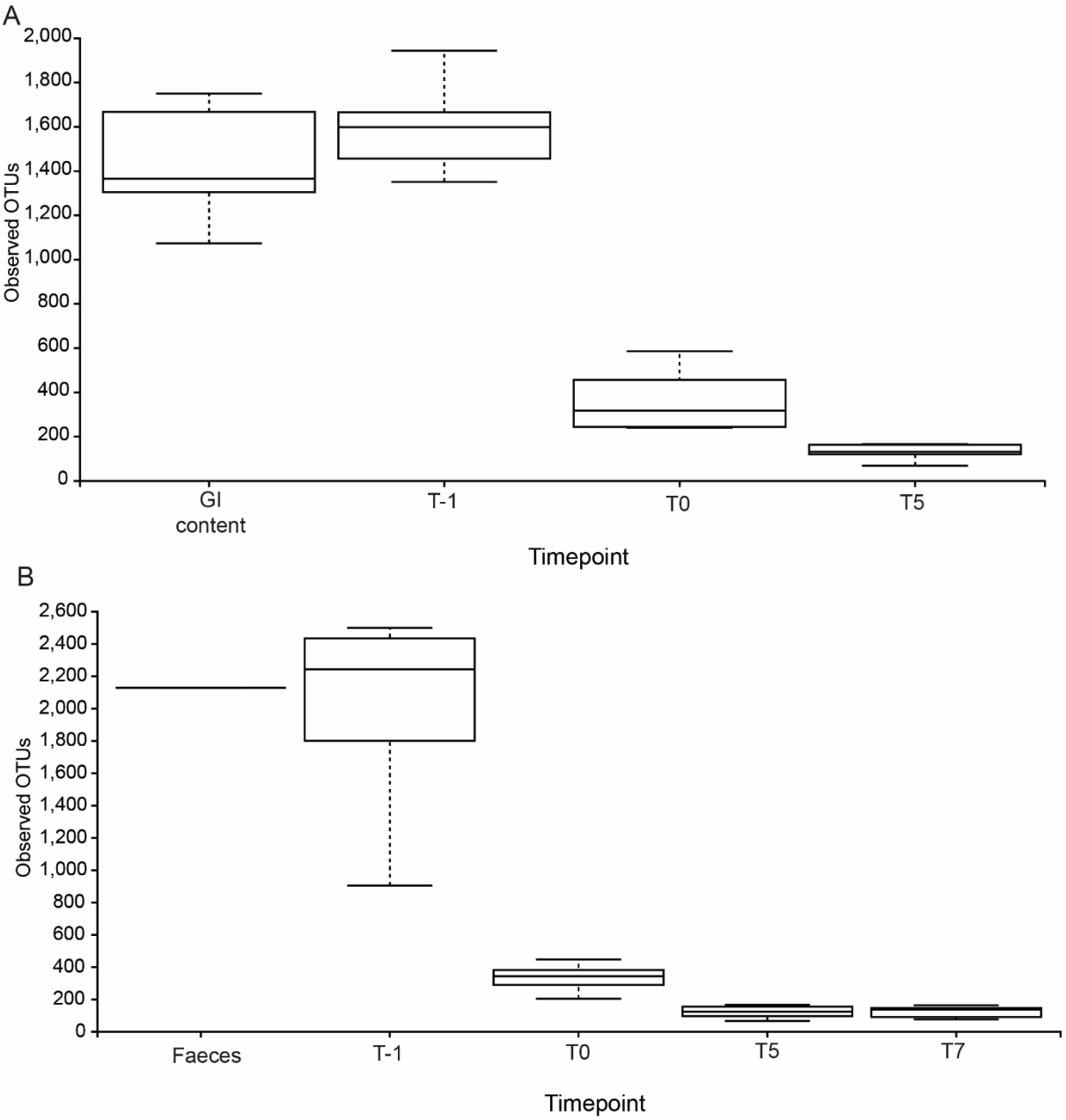


Figure S1: The average percentage of ADF (acid detergent fiber) and starch within the gut content from five areas of the equine gastrointestinal system from three horses (1, 2 and 3 in Table S1). Error bars show standard deviation of the values within each group.



828 **Figure S2:** Comparison of metabolic profiles of *ex* vivo gut content and the gut model
829 at steady state (T5). Multivariate models built with the ^{1}H NMR spectra from samples
830 of GI content and samples from all vessels of the three separate gut models in the
831 concordance study (inoculated with feces from different horses). A PCA model (R^2 =
832 0.64) was built and A) scores plot and B) the loading plot for PC1 (representing 48 %
833 of the total variance in the dataset) was visualised. C) The correlation coefficient plot
834 from the OPLS-DA model built with these spectra (Q^2Y = 0.82). PC, principle
835 component; DEG, diethylene glycol; TMA, trimethylamine.



836 **Figure S3:** Alpha rarefaction boxplots showing bacterial diversity of the
837 gastrointestinal contents/feces and the time points of the gut models of: A) the
838 concordance study and B) the repeatability study.

839

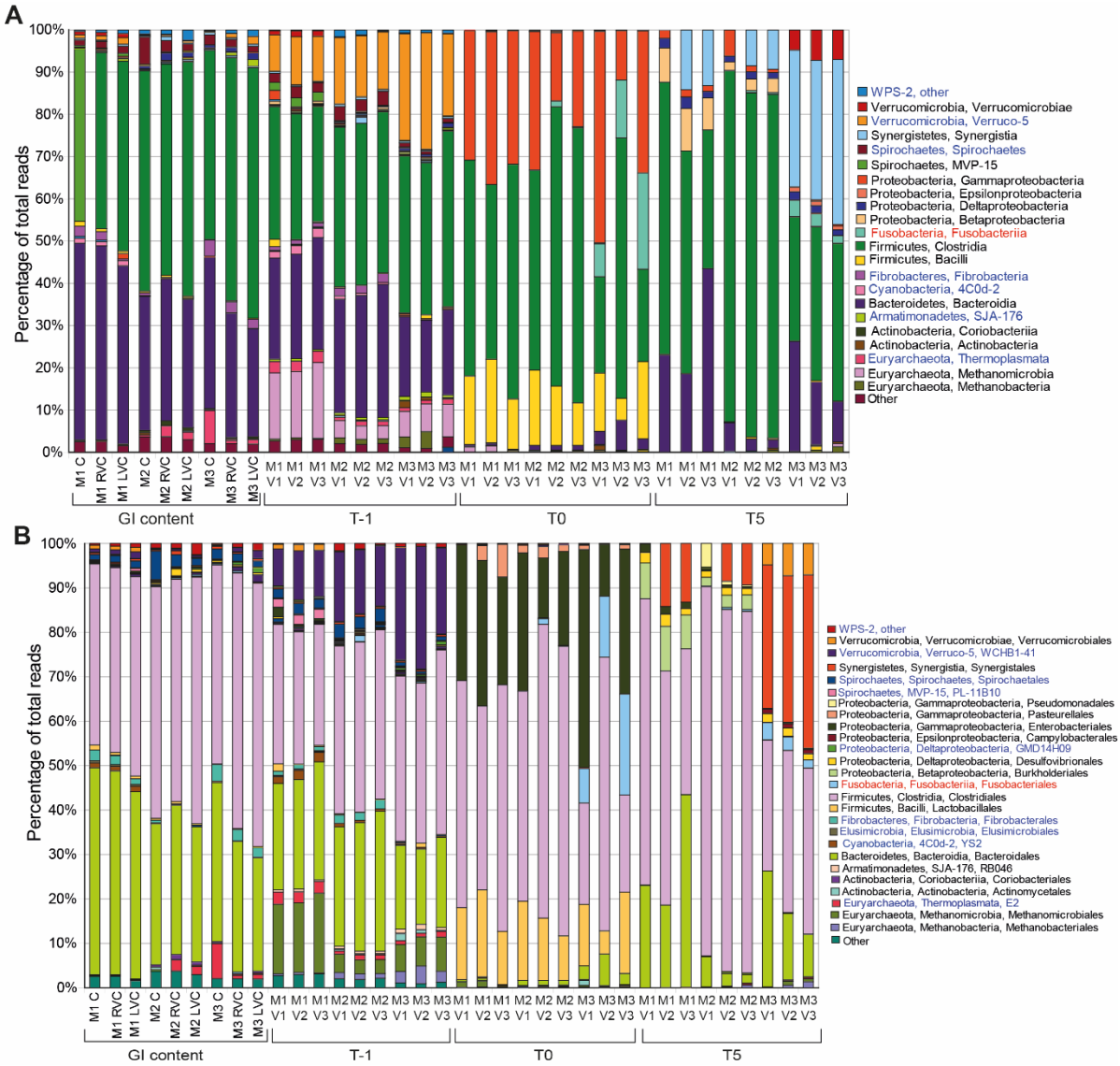


Figure S4: Relative abundance of bacterial A) classes and B) orders identified in samples from the GI content and all vessels the three gut models of the concordance study (inoculated with feces from three different horses). The key indicates whether bacterial phyla are: identified in both gastrointestinal and gut model samples at T5 (black), only in gastrointestinal samples (blue) or only in gut model samples (red). C, cecum; RVC, right ventral colon; LVC, left ventral colon; V, vessel; T, turnover.

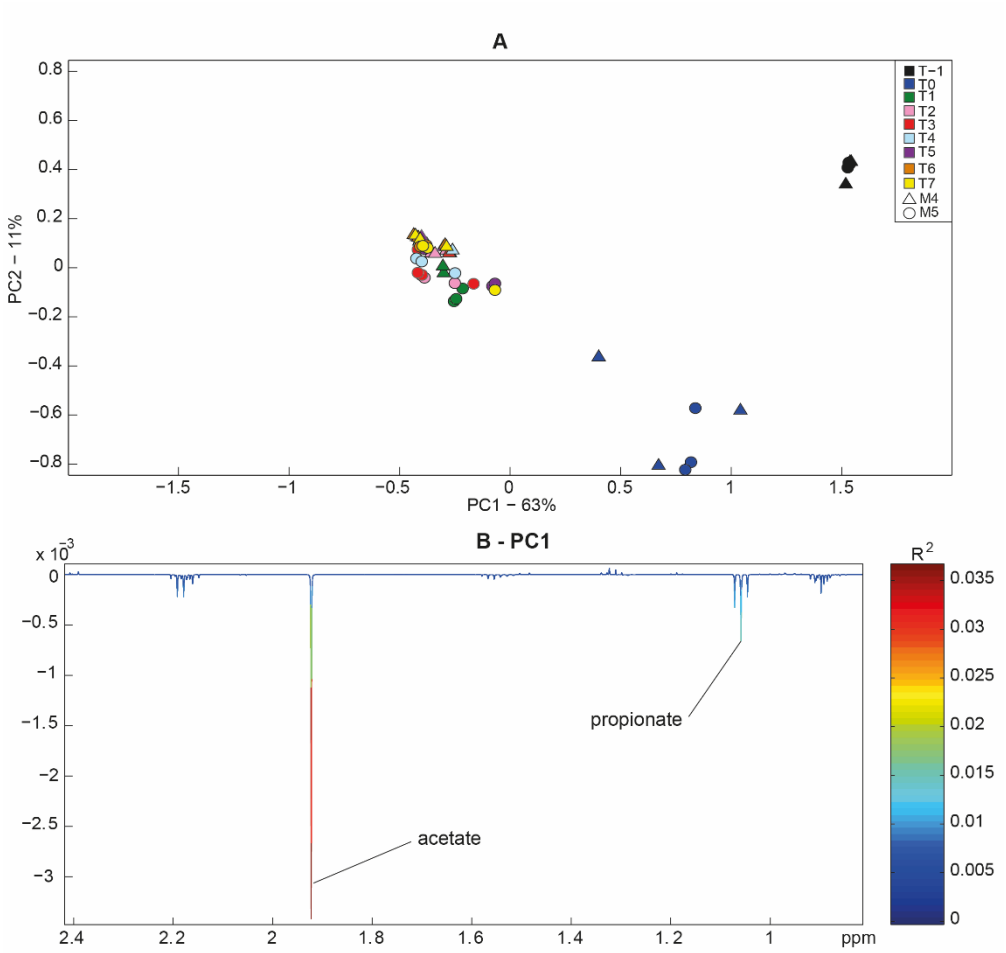
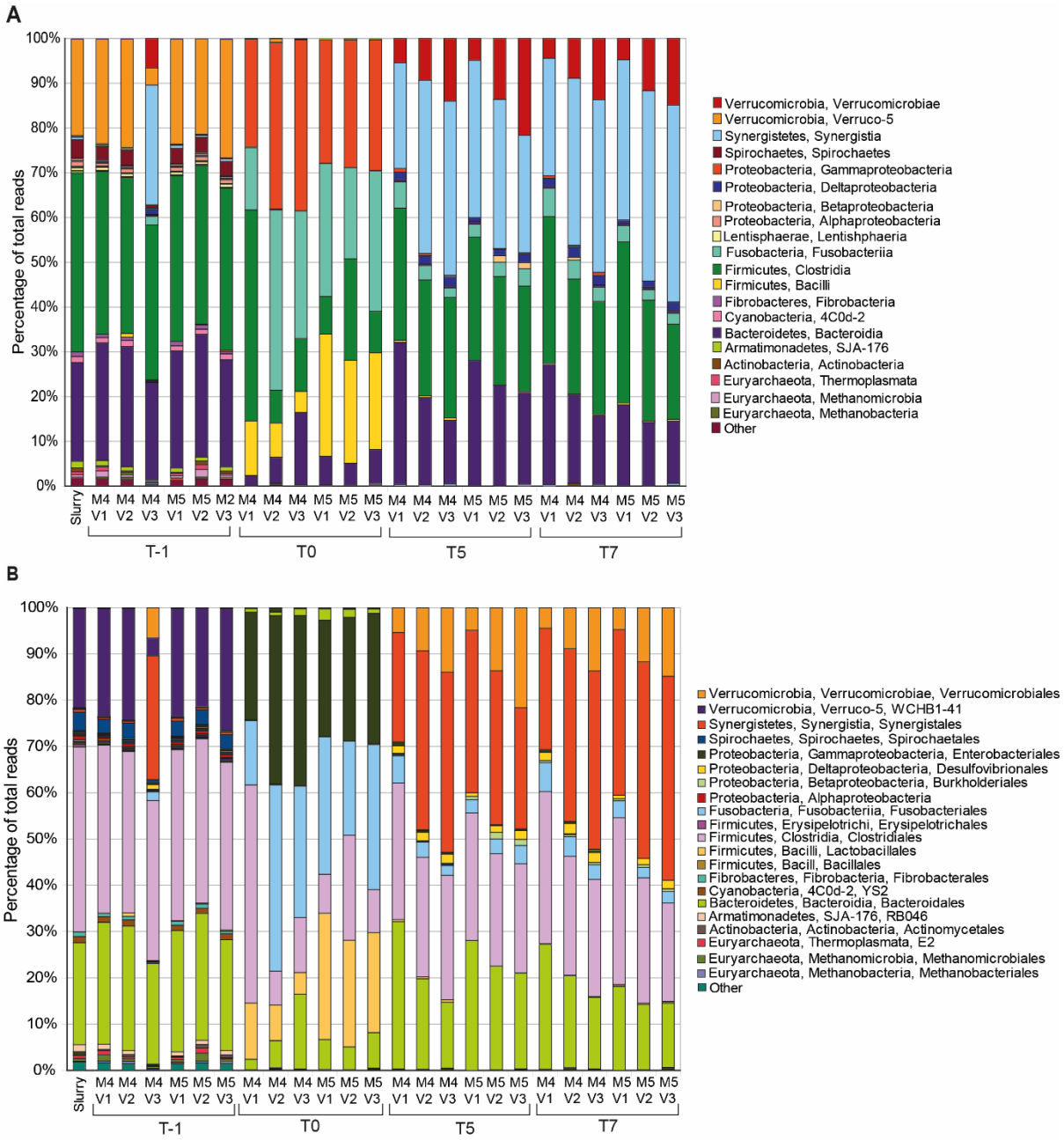


Figure S5: Demonstration of the repeatability of the metabolic profiles of the gut models from the repeatability study. PCA model constructed with the ^{1}H NMR spectra gained from the all vessels of the two gut models inoculated with the feces from the same horse (R^2 = 0.75). A) The PCA scores plot for this model and B) the loading plot for PC1 (representing 63 % of the total variance in the dataset). T, turnover; PC, principle component; M, model.



857 **Figure S6:** Relative abundance of bacterial A) classes and B) orders identified in
858 samples from all vessels the two gut models of the repeatability study (inoculated with
859 feces from the same horse).C, cecum; RVC, right ventral colon; LVC, left ventral colon;
860 V, vessel; T, turnover; M model.