—Original Article—

In vitro analysis of the effect of supplementation with activated charcoal on the equine hindgut

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The present study uses in vitro analytical techniques to investigate the effect of activated charcoal on the microbial community of the equine hindgut and the metabolites they produce. Incubations were performed in Wheaton bottles using a 50 ml incubation of a high-energy feed or a low-energy feed, plus bottles with no added food source, together with five levels of activated charcoal (0, 10, 25, 50 or 100 mg per bottle) and fecal samples as a bacterial inoculum. Using this method the rate of gas production, volatile fatty acid and ammonia concentrations, and pH values were analyzed and found to vary depending on the addition of feed, but the activated charcoal had no effect (P>0.05) on any of these. It is already believed that the effect of activated charcoal as a control for toxic substances is at its highest in the foregut or midgut of animals, and therefore should have little impact on the hindgut. The data presented here suggest that if any of the activated charcoal does reach the hindgut, then it has no significant impact on the microbial community present, nor on the major metabolites produced, and so should not have a detrimental effect on the principal site of fermentation in the horse.

Key words: *activated charcoal, digestive metabolites, horse, microbial profiles*

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Activated charcoal, also known as activated or active carbon, is a porous, carbonaceous material which can be manufactured by the carbonization and activation of biological, organic substances [18]. It has numerous applications and is well utilized in a number of areas of science, including: medicine (therapeutic and detoxification); environment control and treatment; and the food industry (e.g. as a dietary supplement). It has also been shown that incubation with activated charcoal can lead to an increased level of accessible crude protein for some potential fodders [e.g. 3].

Dietary treatments involve using activated charcoal via oral ingestion, rather than intravenously [20], with the theory being its ability to adsorb substances will lead to potentially toxic compounds in an animal's diet being prevented from being absorbed across the gastrointestinal wall [16]. However, there are also potential disadvantages, as activated charcoal has a non-specific absorbency and consequently can bind not only the intended toxins, but can also bind essential nutrients [10] in the gut of animals. In order for the charcoal to work and have a positive effect, it must establish contact with the unwanted target substance in the gastrointestinal tract [7], whilst hopefully not binding to and removing any desirable compounds.

In many species, including the horse, charcoal has been administered to relieve a range of different digestive problems including: endotoxemia [22], colic [19], flatulence [8] and ingested toxins [12]. In addition, some companies suggest that it may be useful in treating colitis and/or laminitis, although there appears to be no scientific publication to support these suggestions. These are all conditions which are seen frequently in the equine veterinary industry and administration of activated charcoal can be regarded as a relatively simple, but apparently effective, treatment. However at present there is limited information and research into the effects of activated charcoal on the microbes of the digestive tract and the consequences, if any, it may have on the microbial community of the digestive tract. There

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has been some speculation that the gut microbiome may be altered in rats [22] as a result of oral introduction of activated charcoal, but this remains speculation. The present study aims to address this lack of information by studying the effect of activated charcoal on the microbial community of the equine hindgut and a number of the metabolites they produce.

Materials and Methods

Animals and collection of fecal material for inoculation of in vitro cultivation vessels

Fecal samples were collected from three adult Welsh Cob geldings (age range four to ten years) stabled at Lluest Equine Yard, Aberystwyth. The animals were all fed the low energy feed (Dodson & Horrell Ltd., Kettering, U.K.–pasture mix–see Table 1 for composition) used for *in vitro* incubations, together with unrestricted access to hay (primarily Timothy). All samples were collected immediately post-defecation, and transferred to the laboratory in capped thermos flasks to maintain the temperature. Samples were pooled in the laboratory to reduce any potential bias effects of samples from a single animal. The pooled sample was used to provide bacterial inocula for *in vitro* cultivation experiments.

Feed preparation for in vitro analysis

Two feedstuffs were used for *in vitro* cultivation studies; a high energy feed (Dodson & Horrell Ltd., competition mix) and a low energy feed (Dodson & Horrell Ltd., pasture mix). The composition of these feeds is shown in Table 1. These feedstuffs were dried in an oven at 60°C for 24 hr to ensure maximum dryness and no moisture. Feed was pre-treated to mimic the condition in which it would reach the hindgut [13, 21]. Initially samples were ground through a screen-grinder (1mm pore size) to mimic the actions of mastication and prehension. For subsequent pre-caecal digestions 2g (pre-digested weight) of the ground dried feeds were used per Dacron bags (5μm pore size). Bags were tied securely during all pre-caecal incubations. The first pre-caecal digestion represented digestion in the stomach and was carried out for 30 min at 38°C in a solution of 2 g/*l* enzymatic pepsin powder (Fisher Scientific, Loughborough, U.K.) and 75 mM HCl. After digestion bags were washed in flowing clean water for 10 min. Bags were then incubated in a solution of 0.1% (w/v) enzymatic pancreatin from porcine pancreas (Sigma, Poole, U.K.), where the solution had been raised to a pH of 8.0 using 5 M NaOH. This incubation lasted 60 min and was performed at 38°C. The pancreatin contained lipase, protease and amylase, thereby allowing digestion of lipids, proteins and starch respectively and was used to replicate digestive conditions during transition of the digesta through the small intestine. The bag was then transferred to a container containing flowing water for 10 min and then oven dried over night at 50°C. For each bottle, 1 g of pre-digested feed was used.

Table 1. Nutritional details of the two different commercial feeds used in this work

	Competition mix	Pasture mix
Est. Digestible Energy MJ/kg	11.5	10.0
Crude Protein (%)	12.0	9.5
Crude Oils and Fats (%)	3.5	4.0
Crude Fiber (%)	11.0	15.0
Crude Ash (%)	8.0	7.0

In addition, the following components were used as ingredients in the two commercial feeds. Competition mix: Oatfeed, Barley, Distiller's Wheat Grains, Wheat, Alfalfa, Extracted Sunflower, Rolled Oats, Cane Molasses, Maize, Wheatfeed, Full Fat Soya, Calcium Carbonate, Salt (1.0%), Unmolassed Sugar Beet, Vegetable Oil, Dicalcium Phosphate, Vitamin/Trace Mineral Premix, Prairie Meal, Magnesium Oxide, L-Lysine. Pasture mix: Wheatfeed, Nutritionally Improved Straw, Barley, Cane Molasses, Wheat, Maize, Limestone Flour, Peas, Oatfeed, Vegetable Oil, Salt, Extracted Sunflower, Mint (0.8%), Vitamin/Trace Mineral Premix, Garlic Granules (0.5%), Dried Carrots (0.5%), Calcined Magnesite, L-Lysine.

Buffer preparation

Artificial saliva [14] was used as a buffer for caecal digestion. This digestion buffer contained: 0.8 g/*l* NH_4HCO_3; 7 g/*l* $NaHCO_3$; 1.89 g/*l* $Na_2HPO_4.12H_2O$; 1.44 g/*l* anhydrous KH_2PO_4; 200 mg/*l* trypticase peptone; 120 mg/*l* $MgSO_4.7H_2O$; 1.32 μg/*l* $CaCl_2.2H_2O$; 1 μg/*l* $MnCl_2.4H_2O$; 0.1 μg/*l* $CoCl_2.6H_2O$; 0.8 μg/*l* $FeCl_3.6H_2O$. While this buffer was developed for work with ruminant samples, and has differences in composition from some reports on horse saliva [1], it has already been used successfully for experiments with horses [13].

To each litre of digestion buffer, 1 m*l* of 1% resazurin solution was added. This solution was made anaerobic by bubbling with carbon dioxide.

Experimental design and in vitro incubations

Incubation work was performed in 150 m*l* Wheaton bottles, with 50 m*l* of buffer in each bottle. Three feed-type conditions were used; Dodson & Horrell Ltd., competition mix (high energy feed), Dodson & Horrell Ltd., pasture mix (low energy feed) or no feed. The high and low energy feeds had been incubated using the pre-caecal digestion treatment steps described above. In addition to the feed conditions, five different charcoal levels (0, 10, 25, 50 or 100 mg per bottle) were factored into the experiment. These quantities of activated charcoal were selected on the basis that their quantity per volume of liquid incubated in the bottles is in keeping *pro rata* with the recommended dose by feed

companies for an average-sized horse (e.g. 30 g of activated charcoal for an animal of around 14–15 hands) relative to the size of the caecum (which is likely to have a volume of around 30 *l*). All incubations were performed in triplicate, giving a total of 45 bottles.

Gas measurements and metabolite analysis

The volumes of gas produced were measured using an ANKOM RF Gas Production Measurement System (Macedon, NY, U.S.A.), with data collected every 5 min from each bottle. After 24 hr one bottle per treatment (i.e. one bottle per triplicate) was removed. From this bottle the pH was recorded and samples were collected for volatile fatty acid (VFA) and DNA analyses. The remaining two bottles per treatment were allowed to incubate for a further 48 hr. At the end of the combined 72 hr these bottles were sampled in the same manner as those opened at 24 hr.

VFA analysis was performed using 4 m*l* of sample from each bottle and mixed with 1 m*l* of 20% orthophosphoric acid containing 4 mM 2-ethyl butyric acid (internal standard). From each tube, 2 m*l* was taken and centrifuged at 13,000 × g for 15 min and then 1.5 m*l* was then syringed into a filter containing a glass fiber pre-filter and a nitrocellulose membrane (0.65 μm pore size). The sample was then filtered into a glass vial and sealed by clamping. This was carried out for each 72 hr sample. Gas chromatography was performed using a Varian CP3380 and autosampler Varian CP8400, using a HP-FFAP column (J & W Scientific, Santa Clara, CA, U.S.A., 10 m length × 0.53 mm I.D. × 1 μm film thickness). The gas chromatography conditions were; inlet/injector temperature of 250°C, 1 μl of sample injected, flame ionization detector temperature at 250°C, carrier gas used was helium at 6 psi (flow 20 m*l*/min), the make-up gas was helium at 20 m*l*/min, hydrogen in the machine was 30 m*l*/min, the air was set at 300 m*l*/min and the split flow was set at 40 m*l* per min. The oven temperatures within the analysis machine were: 80°C initial temperature and 1 min hold after injection, followed by a 20°C per min increase up to 120°C, then 6.2°C/min increase up to 140°C, and finally 20°C/min up to 205°C with a final hold for 5.52 min.

Ammonia levels were determined from 800 μl of the contents of each bottle. Each sample was put into 200 μl 10% [w/v] TCA and centrifuged for 15 min at 14,000 × g and 4°C. In preparation for analysis 400 μl of deionised water was added to 100 μl of the supernatant. A 20 μl sample of this dilution was reacted with: 60 μl of (13 mg/*l* NaOH, 4 mg/*l* EDTA); 100 μl of (10 g/*l* phenol, 50 μg/*l* sodium nitroprusside, 5 g/*l* of NaOH and 15% (v/v) of sodium hypochlorite). Reactions were then performed for 15 min in the dark before the absorbance was measured at 680 nm using a BioTek Epoch Spectrophotometer System (Swindon, U.K.).

DNA extraction and purification

DNA was extracted from one replicate per treatment (the reaction terminated after 24 hr). Flasks were swirled to mix the content before extraction, which was and samples were extracted using QIAamp® DNA Mini Stool Kits (Qiagen Ltd., West Sussex, U.K.). The manufacturer's instructions were followed with the exception of increasing the initial incubation temperature from 70 to 95°C for 5 min (as suggested by the manufacturers), which was found to give better yields from samples potentially containing a high number of Gram-positive bacteria and has been used previously with samples from horses [4].

Following extraction, the concentration and purity of the DNA was assessed using a BioTek Epoch Spectrophotometer System to measure the absorbance at 260 nm, as well as the A_{260}:A_{280} ratio.

PCR amplification

A partial fragment of the 16S *rRNA* gene was amplified with the bacterial primers *27F* (5′-AGA GTT TGA TCC TGG CTG AG-3′) and *1389R* (5′-AGG GGG GGT GTG TAG AAG-3′). These primers have been described as being able to amplify DNA from a diverse range of bacteria [9, 23]. The 27F primer had a Cy5-label attached to facilitate its use for terminal restriction fragment length polymorphism (TRFLP) analysis. PCR was performed in a final volume of 25 μl, which contained 0.25 μl of each primer (50 μM stock concentration), 1 μl of template DNA, 12.5 μl ImmoMix^{TM} (Bioline, London, U.K.) and 11 μl of molecular grade water.

Amplification was performed with the following steps: 95°C hot start for 10 min; 25 cycles (94°C for 1 min, 55°C for 1 min and 72°C for 1 min); 1 cycle (94°C for 1 min, 55°C for 1 min and 72°C for 5min) and a final hold at 4°C. Successful amplification was verified by electrophoresis on a 1% (w/v) agarose gel to detect amplicons of the anticipated length; around 1,300–1,400 base pairs (bp).

TRFLP analysis

PCR products were digested using restriction enzymes *Hae* III, *Hha* I and *Msp* I (New England Biolab, Hitchin, U.K.) in preparation for TRFLP analysis. Reactions were performed with a single enzyme per reaction, using 2.5 U for *Hha* I and 1.25 U for *Msp* I and *Hae* III. For the reactions 50 ng of amplicon sample, the manufacturer's buffer and molecular grade water (final volume of 50 μl) were used and were performed at 37°C for 5 hr. Samples were cleaned by ethanol precipitation.

TRFLP analysis was performed on a CEQ^{TM} 800 Genetic Analysis capillary sequencer (Beckman Coulter, High Wycombe, U.K.). Internal size standard 600 (Beckmann Coulter) DNA was used to determine the sizes of the fluorescently labelled fragments within the size range 60–600

bp. Analysis was restricted to only those terminal restriction fragments which were higher than 0.5% of the maximum value and samples were compared by converting all peaks to percentages of the total for a particular sample. Inter-sample comparisons were performed using canonical analysis of principal coordinates [2].

Statistical analyses

Cumulative gas production levels obtained from the ANKOM RF Gas Production system at were fitted to the exponential equation using the Neway Excel curve-fitting program, Fit Curve, as described by Ørskov and McDonald [17], and "a" (the gas production from the readily fermented fraction), "b" (the gas production from the slowly fermented fraction) and "c" (fermentation rate) values calculated.

Variations in the cumulative amounts of gas production were assayed by analysis of variance (ANOVA) for differences in feed type (high energy, low energy, or no feed), charcoal level, and feed × charcoal interactions using samples from the following time points: 3, 6, 12, 16, 24, 36, 48 and 72 hr. In addition, the equivalent ANOVA comparison was repeated for only high energy versus low energy feeds (i.e. without the bottles containing on the fecal inoculum). End point pH values, VFA levels and ammonia levels were also analysed by the same methods. Comparisons between individual pairs of feed-charcoal combinations were performed by Tukey's analysis.

Inter-sample comparisons between TRFLP profiles were performed using canonical analysis of principal coordinates [2]. Plots were shown using the two principal components as axes.

Results

Gas production

The volumes of gas produced are shown in Table 2. Based on ANOVA of the three feed categories (high energy, low energy and no feed), there was a significant difference ($P<0.001$) in the amount of gas produced based on the type of feed being used. This was also true when ANOVA was performed to compare the high energy versus low energy feeds, where there were significant differences ($P<0.05$) observed in terms of gas production from 6 hr onwards. However, there were no significant differences between the different levels of activated charcoal, and likewise, no global differences between the interactions between the type of feed and the activated charcoal levels.

VFA production

The concentrations of volatile fatty acids (VFAs) produced are shown in Table 3. Total VFAs, acetic acid, propionic acid and butyric acid levels are influenced by feed ($P<0.001$) in the comparisons between high energy, low energy and no feed, but was not by charcoal levels, or a feed × charcoal interaction ($P>0.05$). No significant difference was seen between any of the samples for valeric acid levels ($P>0.05$). ANOVA comparisons between bottles with high and low energy feeds failed to show significant differences in terms of either total VFA production or acetic acid production, but significant differences were observed between butyric acid and propionic acid levels.

Ammonia concentrations

The concentrations of ammonia produced are shown in Table 4. These values were influenced by the feed in the incubation ($P<0.001$), but not by charcoal levels, or a feed × charcoal interaction ($P>0.05$).

PH values

No significant difference ($P>0.05$) in pH value was detected between the samples from either the high energy or the low energy feed for both samplings times, with respective values of 6.23 (± 0.05) and 6.24 (± 0.01) for the 24 hr samples and 6.27 (± 0.01) and 6.27 (± 0.01) for the 72 hr samples. However, both were significantly different ($P<0.001$) from those samples from the tubes with no feed added, which had values of 6.76 (± 0.04) and 6.87 (± 0.05) for the 24 hr samples and 72 hr samples respectively. No difference ($P>0.05$) in pH value based on the different levels of charcoal.

TRFLP analysis

Figure 1 shows the plot of the two principal coordinates, which together account for over 75% of the variation observed in the various samples. A cluster at the bottom left of the figure indicates all of the samples where no feed source (O1–O5) was added at the outset of the *in vitro* incubations. No other clusters, either based on feed content or charcoal supplementation, could be identified.

Discussion and Conclusions

As expected, the gas production values in Table 2 demonstrate a difference between gas production levels depending on which feed was being used to set up the incubations. For example, in all cases the value of "a" (i.e. gas production from the immediately soluble fraction) was low, including some cases of negative values. This observation of a negative "a" value is in keeping with previous reports [5, 15] and is indicative of delayed microbial colonization of the substrate during the early incubation stages. In the case of bottles which had a feed added to it, there was evidence, "b" values, of gas production from the immediately insoluble fraction, and in the case of bottles with no feed added there

Table 2. Mean volume of gas produced at different time points for each reaction

	Activated charcoal (mg) for high energy feed					Activated charcoal (mg) for low energy feed					Activated charcoal (mg) for no feed				
	0	10	25	50	100	0	10	25	50	100	0	10	25	50	100
3 hr	2.1 ^{x} (0.03)	1.9 ^{xy} (0.10)	2.2 ^{x} (0.01)	2.2 ^{x} (0.43)	1.9 ^{x} (0.13)	2.2 ^{x} (0.01)	1.9 ^{x} (0.12)	2.1 ^{x} (0.01)	2.0 ^{x} (0.16)	2.0 ^{x} (0.13)	0.4 ^{y} (0.24)	0.5 ^{y} (0.04)	0.2 ^{y} (0.19)	0.4 ^{y} (0.21)	0.7 ^{y} (0.01)
6 hr	3.3 ^{x} (0.44)	3.2 ^{x} (0.13)	3.4 ^{x} (0.01)	3.5 ^{x} (0.00)	3.1 ^{x} (0.12)	3.3 ^{x} (0.10)	2.9 ^{x} (0.11)	3.2 ^{x} (0.02)	3.0 ^{x} (0.14)	3.0 ^{x} (0.14)	0.8 ^{y} (0.24)	0.7 ^{y} (0.05)	0.5 ^{y} (0.18)	0.8 ^{y} (0.20)	1.0 ^{y} (0.02)
12 hr	6.4 ^{x} (0.11)	6.0 ^{x} (0.03)	6.9 ^{x} (0.03)	5.5 ^{x} (1.23)	6.2 ^{x} (0.34)	5.8 ^{x} (0.02)	5.6 ^{x} (0.16)	5.8 ^{x} (0.06)	5.7 ^{x} (0.23)	5.7 ^{x} (0.26)	0.9 ^{y} (0.23)	1.0 ^{y} (0.05)	0.6 ^{y} (0.17)	1.0 ^{y} (0.21)	1.0 ^{y} (0.24)
24 hr	9.5 ^{x} (0.13)	9.2 ^{x} (0.09)	10.2 ^{x} (0.16)	8.9 ^{x} (1.49)	9.4 ^{x} (0.36)	8.8 ^{x} (0.06)	8.8 ^{x} (0.05)	9.2 ^{x} (0.27)	8.9 ^{x} (0.36)	8.8 ^{x} (0.32)	0.9 ^{y} (0.23)	1.0 ^{y} (0.12)	0.7 ^{y} (0.14)	1.0 ^{y} (0.21)	1.1 ^{y} (0.03)
36 hr	10.5 ^{x} (0.15)	10.7 ^{x} (0.19)	11.7 ^{x} (0.26)	9.6 ^{x} (2.09)	10.6 ^{x} (0.37)	10.6 ^{x} (0.13)	10.3 ^{x} (0.09)	10.9 ^{x} (0.42)	10.6 ^{x} (0.37)	10.5 ^{x} (0.34)	1.2 ^{y} (0.20)	1.3 ^{y} (0.10)	1.1 ^{y} (0.12)	1.2 ^{y} (0.18)	1.4 ^{y} (0.03)
48 hr	10.9 ^{x} (0.15)	11.3 ^{x} (0.1)	12.4 ^{x} (0.32)	10.3 ^{x} (1.96)	11.0 ^{x} (0.40)	11.5 ^{x} (0.06)	10.8 ^{x} (0.11)	11.6 ^{x} (0.40)	11.3 (0.36)	11.4 ^{x} (0.32)	1.4 ^{y} (0.20)	1.4 ^{y} (0.16)	1.2 ^{y} (0.11)	1.3 ^{y} (0.20)	1.6 ^{y} (0.80)
60 hr	11.1 ^{x} (0.15)	11.6 ^{x} (0.10)	12.7 ^{x} (0.35)	10.5 ^{x} (1.94)	11.2 ^{x} (0.53)	11.9 ^{x} (0.08)	11.0 ^{x} (0.06)	11.9 ^{x} (0.45)	11.6 ^{x} (0.37)	11.9 ^{x} (0.23)	1.5 ^{y} (0.20)	1.5 ^{y} (0.19)	1.3 ^{y} (0.10)	1.4 ^{y} (0.21)	1.7 ^{y} (0.11)
72 hr	11.1 ^{x} (0.17)	11.7 ^{x} (0.07)	12.8 ^{x} (0.38)	10.6 ^{x} (1.98)	11.3 ^{x} (0.56)	12.1 ^{x} (0.10)	11.1 ^{x} (0.87)	12.0 ^{x} (0.48)	11.9 ^{x} (0.40)	12.2 ^{x} (0.26)	1.5 ^{y} (0.20)	1.5 ^{y} (0.20)	1.3 ^{y} (0.10)	1.4 ^{y} (0.22)	1.8 ^{y} (0.14)
a	–0.166	0.019	0.002	–0.317	–0.177	–0.098	0.056	–0.088	–0.090	0.104	–0.028	–0.449	–0.259	–0.063	–0.321
b	7.47	16.85	9.95	13.13	12.91	12.47	12.87	12.70	11.80	11.51	0.87	0.95	1.06	0.98	1.06
c	1.62	0.99	0.93	1.35	1.39	1.21	1.20	1.20	1.33	1.36	9.15	10.22	10.46	13.65	11.27

Values are expressed as m*l* of gas per gram of dry matter feed. Values within a row that share a superscript are not significantly different (P>0.05), based on Tukey's values.The entries for "a", "b" and "c" denote the gas production from the readily fermented fraction, gas production from the slowly fermented fraction, and fermentation rate, respectively.

Table 3. Mean concentration of volatile fatty acids produced at the end of each reaction with standard error values shown in parenthesis

	Activated charcoal (mg) for high energy feed					Activated charcoal (mg) for low energy feed					Activated charcoal (mg) for no feed				
	0	10	25	50	100	0	10	25	50	100	0	10	25	50	100
Total	61.0 ^{x} (0.60)	65.8 ^{x} (3.15)	62.4 ^{x} (0.83)	63.1 ^{x} (0.65)	59.8 ^{x} (1.22)	61.2 ^{x} (1.16)	60.8 ^{x} (2.06)	59.3 ^{x} (1.38)	59.1 ^{x} (1.69)	58.6 ^{x} (1.60)	19.3 ^{y} (1.25)	21.4 ^{y} (0.43)	20.5 ^{y} (0.25)	21.0 ^{y} (0.61)	21.0 ^{y} (0.19)
Acetic	38.5 ^{x} (0.59)	42.1 ^{x} (2.11)	40.3 ^{x} (0.56)	40.7 ^{x} (0.59)	38.0 ^{x} (0.61)	40.3 ^{x} (0.93)	39.1 ^{x} (1.37)	38.1 ^{x} (1.05)	38.2 ^{x} (1.07)	38.8 ^{x} (1.23)	12.7 ^{y} (0.86)	14.0 ^{y} (0.33)	13.5 ^{y} (0.19)	13.9 ^{y} (0.38)	13.9 ^{y} (0.20)
Propionic	16.5 ^{x} (0.07)	16.9 ^{x} (0.75)	16.0 ^{x} (0.14)	16.3 ^{x} (0.10)	15.8 ^{x} (0.64)	15.0 ^{x} (0.23)	16.0 ^{x} (0.51)	15.5 ^{x} (0.35)	15.3 ^{x} (0.56)	14.2 ^{x} (0.30)	3.6 ^{y} (0.38)	4.3 ^{y} (0.07)	4.1 ^{y} (0.07)	4.1 ^{y} (0.19)	3.9 ^{y} (0.09)
Butyric	4.6 ^{x} (0.05)	5.2 ^{x} (0.27)	4.8 ^{x} (0.09)	4.7 ^{x} (0.09)	4.6 ^{x} (0.13)	4.6 ^{x} (0.09)	4.5 ^{x} (0.16)	4.4 ^{x} (0.04)	4.4 ^{x} (0.08)	4.4 ^{x} (0.06)	2.0 ^{y} (0.03)	2.0 ^{y} (0.03)	1.9 ^{y} (0.01)	2.1 ^{y} (0.02)	2.1 ^{y} (0.05)
Valeric	1.4 (0.02)	1.5 (0.09)	1.4 (0.06)	1.4 (0.03)	1.3 (0.02)	1.3 (0.05)	1.3 (0.04)	1.2 (0.05)	1.2 (0.01)	1.2 (0.04)	1.0 (0.02)	1.0 (0.01)	0.9 (0.01)	1.0 (0.02)	1.1 (0.05)

Values are expressed as mM concentrations. Values within a row that share a superscript are not significantly different (P>0.05), based on Tukey's values. Valeric acid values did not vary between samples.

Table 4. Mean concentration of ammonia produced at the end of each reaction

	Activated charcoal (mg)				
	0	10	25	50	100
High energy feed added	38.5 ^{a} (0.59)	42.1 ^{a} (2.11)	40.3 ^{a} (0.56)	40.7 ^{a} (0.59)	38.0 ^{a} (0.61)
Low energy feed added	40.3 ^{a} (0.93)	39.1 ^{a} (1.37)	38.1 ^{a} (1.05)	38.2 ^{a} (1.07)	38.8 ^{a} (1.23)
No feed	12.7 ^{b} (0.86)	14.0 ^{b} (0.33)	13.5 ^{b} (0.19)	13.9 ^{b} (0.38)	13.9 ^{b} (0.20)

Values are expressed as (N mmol/*l*) concentrations. From ANOVA calculations, differences between samples with feed were significantly different (P<0.001) but no differences (P>0.05) were detected for different levels of charcoal. Values that share the same superscript are not significantly different (P>0.05).

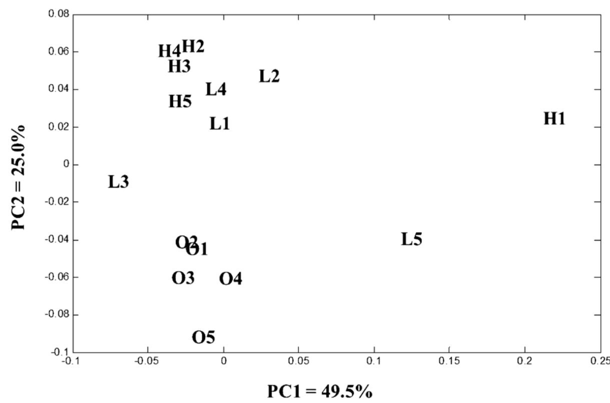

Fig. 1. Plot of a pair wise comparison of the principal coordinate (PC1) and the next largest coordinate (PC2) following canonical analysis of principal coordinates. The letter denotes either feeding with a high energy diet (H), low energy diet (L) or no food added (O). The numerical label shows the level of charcoal added per bottle (0, 10, 25, 50 or 100 mg, denoted by 1, 2, 3, 4 and 5 respectively). The data represent incubations with *Hha* I.

was a higher "c" value, indicating gas production due to the insoluble fraction. However, no significant differences were detected based on the quantity of charcoal which was present in each bottle, suggesting that the presence, and level, of charcoal has no impact on the volume of gas production as a result of food-type (high or low energy) being digested.

This pattern was repeated when the production of metabolites was investigated, with significant differences observed between bottles incubated with a source of food, relative to those with no food added, but no significant differences based on activated charcoal levels. For example, the production of VFAs was influenced by the presence or absence of a food source. Specifically, the abundance of the three major VFAs (acetic, butyric and propionic) was influenced by the addition of a food source, with differences in the butyric and propionic values also being influenced by the type of feed added. However, no significant difference was detected ($P>0.05$) between samples based on their charcoal content, suggesting that the presence of the charcoal does not influence the production of VFAs. Similarly the level of ammonia produced was influenced by the presence or absence of a food source ($P<0.001$) but not by the activated charcoal level. The change in metabolites between food being added, or not, was also associated with a drop in the pH when food was present relative to no food being added ($P<0.001$), but no difference ($P>0.05$) in pH was detected between charcoal levels.

Based on using incubations with microbes from fecal samples as a representation of microbes that have recently been living in the hindgut of the horse, the current study suggests that the addition of activated charcoal to this population does not make a significant impact on the fermentation rate of food, or on a number of the major metabolites (e.g. VFAs, ammonia) produced from this fermentation. Moreover, a plot derived from data using TRFLP data from bacterial 16S *rRNA* genes (Fig. 1) failed to discriminate between bacterial populations relative to charcoal concentrations, suggesting that any effects detected in the present study are secondary in significance relative to those seen in terms of the energy content of the food source. Thus, this *in vitro* experiment suggests that there is little impact of activated charcoal on the microbial population, the fermentation carried out by these microbes, or the metabolites they produce.

It may be argued that information gained from *in vitro* analyses provide limited information due to the challenges of trying to replicate the conditions of the equine digestive tract. However the present study attempts to replicate conditions found in the pre-caecal areas of the tract prior to the incubations: mastication; acidic conditions of the stomach; and the digestive potential of proteins, lipids, and simple carbohydrates in the small intestine. Indeed, there is a counter-argument based on the observations that although there is relative stability of the microbial population at the level of an individual horse, there is still an appreciable day-to-day variation for samples collected from individual horses [4]. Therefore, performing *in vitro* analyses provides ideal conditions, for controlled conditions which reduce the potential for differences to arise due to either temporal variation, or animal-to-animal variation.

These observations suggest that the activated charcoal that reaches the hindgut, is unlikely to impact on the activities carried out in this region of the digestive tract. However, it is worth noting that, at least in medical research, the use of activated charcoal as a toxin decontaminator has focused on the foregut [6], meaning that the effect of activated charcoal may be most extensively observed prior to the hindgut. Interestingly, charcoal has been shown to affect the metabolites produced using *in vitro* analysis with rumen samples [11]. However, in that particular example, charcoal levels were much higher than those used in the present study (where we have used levels in keeping with those recommended by the suppliers who sell activated charcoal for use in the horse). It is also worth noting that in the rumen simulation there were no acidic treatments necessary, as the acidic conditions of the abomasum lie distal to the main fermentation sites of the rumen and reticulum.

In conclusion, we observed that activated charcoal had no detectable effect on the microbial community in the hindgut of the horse, either in terms of the population dynamics (based on TRFLP data), gas production levels, or the major

metabolites produced (VFAs and ammonia). Assuming the presence of any effects in the foregut or midgut, then it can be concluded that any residual unreacted charcoal molecules that reach the hindgut are not likely to be detrimental to the fermentative functioning of the hindgut.

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