

# Changes in faecal bacteria associated with concentrate and forage-only diets fed to horses in training

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## Summary

**Reasons for performing study:** Diets rich in readily fermentable carbohydrates, fed traditionally to meet the increased energy requirements of the performance horse, are associated with a number of gastrointestinal disorders that involve disturbances in the intestinal microbiota, however, these changes are poorly understood.

**Objectives:** With the long-term objective of improving intestinal health and to increase understanding of the relationship between diet and microbiota, the effect of feeding Standardbred horses a high-energy forage-only (F) diet was studied compared to a more traditional forage-concentrate (C) diet on faecal microbiota.

**Methods:** Diets were fed in a cross-over design to 6 mature geldings on a scheduled training regime, both periods consisting of 29 days. DNA was extracted from faecal samples collected at 4 time points from each period, bacterial 16S rRNA genes were amplified and community composition assessed by terminal-restriction fragment length polymorphism, cloning and sequencing. Faecal pH and cultivable lactic acid bacteria (LAB) and enterobacteria were also assessed on the final collection day of each period.

**Results:** Diet F resulted in a microbial composition that was more stable between sampling periods and had lower counts ( $P < 0.05$ ) of cultivable LAB and specifically members of the *Streptococcus bovis/equinus* complex. Motile and swarming *Lactobacillus ruminis* was present in all horses on diet C and not in horses on diet F. Diet C also resulted in the increase ( $P < 0.05$ ) in members of *Clostridiaceae* cluster III and a concomitant reduction ( $P < 0.05$ ) in an unknown group of *Bacteroidales*.

**Conclusions and potential relevance:** The greater microbial stability and reduction in LAB and members of the *Streptococcus bovis/equinus* complex on diet F indicate an opportunity to develop feeding strategies that support equine health and welfare. Novel changes identified in the faecal microbiota that resulted from carbohydrate inclusion merit further investigation.

## Introduction

The gastrointestinal tract of the horse is adapted to the continuous grazing of a fibre-rich diet. However, a diet rich in readily

fermentable carbohydrates has been adopted in an attempt to meet the increased energy requirements of the modern performance horse. Unfortunately, overload of readily fermentable carbohydrates results in the onset of a number of important equine diseases including colic (Wrangel 1911) and laminitis (Clarke *et al.* 1990). Although the exact aetiology has not been defined, it is generally accepted that some forms of colic and laminitis may be the result of changes in the microbial population and fermentation profile in the hindgut (Bailey *et al.* 2004).

There are a number of studies that have observed the effect of readily fermentable carbohydrate inclusion in the diet on culturable bacteria in the equine gut (de Fombelle *et al.* 2003; Varloud *et al.* 2007; Respondek *et al.* 2008). However, studies on human faecal samples showed that up to 80% of microbial species inhabiting the gastrointestinal tract cannot be cultured (Suau *et al.* 1999). Few studies of the horse microbiota have taken advantage of the culture independent molecular based approaches, and the majority of such studies have focused on the oligofructose (OF) model of laminitis (Milinovich *et al.* 2007, 2008).

In the OF model of laminitis it has been observed that a substantial increase in lactic acid bacteria (LAB), particularly *Streptococcus* spp. precedes disease initiation (Milinovich *et al.* 2008). When *Streptococcus* spp. and other LAB become abundant, lactate production becomes excessive (Medina *et al.* 2002) resulting in a substantial reduction in pH (Julliand *et al.* 2001). It is commonly believed that the reduction in pH leads to the increased microbial production of amines that, when absorbed by the host in excessive amounts, may cause laminitis (Crawford *et al.* 2007). Although changes in the microbial population resulting from OF overload have been well characterised, an understanding of the effects of carbohydrates on the intestinal microbiota is needed.

Excessive intake of OF and consequent laminitis are not common in the management of performance horses, although excessive intake of starch and the development of colic are. Current recommendations suggest a maximum of 2–4 g starch/kg bwt/meal (Kienzle 1994). The aim of the present experiment was to investigate the effect of feeding a high-energy forage-only (F) diet as compared to a forage-concentrate (C) diet on the composition of the faecal microbial community of the performance horse. The microbial community was assessed by means of terminal-restriction fragment length polymorphism (T-RFLP), cloning and sequencing, and culture of LAB and enterobacteria. By using both culture based and molecular profiling methods, a wider and more accurate picture of the effects of carbohydrate inclusion was expected.

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## Materials and methods

### Animals and management

This study was approved by the Uppsala local ethics committee. Six mature Standardbred trotter geldings (age mean  $\pm$  s.d. 6.5  $\pm$  0.4 years, weight 515  $\pm$  21 kg) were studied. All horses were in race training and trained regularly on a track every third to fourth day throughout the experimental periods. Horses were fed *diets F* and *C* in a cross-over design, over two 29 day periods. *Diet F* was comprised of an early cut timothy/meadow fescue haylage, while *diet C* comprised a late cut timothy/meadow fescue (50% of dry matter) and concentrate (35.8% starch, 50% of DM) (82% oats, 14% soy bean meal, 2.7% wheat bran and 1.4% sugar). Both diets were supplemented daily with 51  $\pm$  2 g of vitamin and mineral supplementation (Miner Röd)<sup>1</sup> and 36  $\pm$  1 g sodium chloride; 34  $\pm$  1 g ground chalk was added to the concentrate diet. The amount of forage fed ranged from 13–17.4 kg/day on *diet F* and from 6.3–8.4 kg/day on *diet C* respectively, according to horse size. The concentrate ration was divided into 3 equal meals/day for a total of 6.3–8.5 kg/day (1.5 g/kg bwt). Proximate analysis of the feedstuffs is shown in Table 1. Feed-allowances were calculated to be isocaloric (116  $\pm$  5 in the haylage-diet and 117  $\pm$  5 MJ metabolisable energy/day in the concentrate diet) and isonitrogenous (1002  $\pm$  45 in the haylage-diet and 1008  $\pm$  45 g digestible crude protein/day in the concentrate-diet) on a daily basis.

### Sample collection

Faecal samples from all of the horses were collected in the morning of Days 7, 14, 21 and 29 in each experimental period. Samples from Days 7, 14 and 21 were put into plastic bags individually and placed in a -20°C freezer directly after collection. Samples from Day 29 were transported immediately to the laboratory for measurement of pH and selective plating and subsequently frozen at -20°C.

### Faecal pH

A subsample of 5 g of each fresh faecal sample collected on Day 29 in both periods was diluted in 20 ml water and the pH was measured (PHM 92)<sup>2</sup>.

### Selective culture of LAB and enterobacteria

From each sample collected on the final day of each period (Day 29), 10 g of faeces were diluted serially ( $10^{-1}$  to  $10^{-9}$ ) in peptone water, containing 0.2% peptone and 0.05% TWEEN and homogenised with a laboratory blender (Stomacher 400)<sup>3</sup> and plated on selective media. LAB were enumerated by plating on Rogosa agar<sup>4</sup> and incubated under anaerobic conditions at 37°C for 24 h. Enterobacteria were enumerated by plating on violet red bile glucose (VRBG) agar<sup>5</sup> cultured aerobically at 37°C for 24 h.

### Identification of LAB and enterobacteria

For each horse in each period colonies growing on Rogosa and VRBG plates (4 per media) were identified by amplification and sequencing of 16S rRNA genes, for a total of 96 colonies. Colony PCRs were performed using broad-range bacterial primers Bact-8F

and 926r (Edwards *et al.* 1989; Muyzer *et al.* 1993). PCR products were purified (QIAquick)<sup>6</sup> and sequenced from Bact-8F. Sequences were checked for quality and nearest match was found using SEQMATCH in RDP.

### Terminal-restriction fragment length polymorphism (T-RFLP)

To obtain a profile of the faecal microbiota, total extracted DNA was analysed by T-RFLP as previously described (Dicksved *et al.* 2008). Briefly, DNA was isolated from 250 mg of all 48 samples (6 horses and 8 sampling days) using a commercial DNA extraction kit (FastDNA SPIN for Soil)<sup>7</sup>. Bacterial 16S rRNA genes were amplified with Bact-8F, 5'-end labelled with 6-carboxyfluorescein and 926r. The PCR was carried out in duplicate and PCR products were digested with a restriction enzyme (*Hae*III)<sup>8</sup>. Digested products were separated on a capillary sequencer (ABI 3730)<sup>9</sup> and T-RFLP profiles were processed using Peak Scanner V1.0. Relative peak area of terminal restriction fragments (TRFs) corresponding to sizes between 35 and 500 base pairs were calculated by dividing individual peak area by total peak area within this size constraint. Only peaks representing a minimum of 0.5% of the summed peak area were included in further analyses.

### Cloning and sequencing

To aid in the identification of bacteria that corresponded to TRFs of interest and to give some sequence information about abundant organisms, small clone libraries from 3 horses on *diet C* and 3 horses on *diet F* were created. Extracted DNA was amplified using primers Bact-8F and 926r. PCR products were gel-purified, cloned (TOPO TA pCR 4.0)<sup>10</sup> and transformed into *E. coli* (TOP10 chemically competent)<sup>10</sup>. A total of 86 clones were sent for bidirectional sequencing and resulting quality sequences were compared to the GenBank database using BLAST search. Sequences were checked for chimeras using Chimera Check version 2.7, and deposited in GenBank at NCBI under accession numbers FJ493072 to FJ493138. Clones were subjected to T-RFLP as described above to facilitate the correlation of sequences to TRFs.

### Phylogenetic analysis of 16S rRNA gene sequences

Sequences were aligned using the hidden Markov model alignment algorithm implemented in the ARB phylogenetic analysis program (Ludwig *et al.* 2004). The alignment model was generated from all available full-length 16S rRNA sequences from type strains (5160 as of August 2008) that were aligned by secondary structure in the Ribosomal Database Project server (Cole *et al.* 2007), then imported into ARB. Cloned sequences were then aligned to the model and inspected manually for alignment errors. For phylogenetic analysis, representative sequences from the orders *Bacteroidales* and *Clostridiales* were included in the analysis, along with a smaller proportion of sequences from more distantly

**TABLE 1: Proximate analysis of feed constituents fed to horses on high-energy forage-only (F) and forage/concentrate (C) diets**

Feed ingredient	Diet	DM (%)	Metabolisable energy (MJ/kg DM)	CP (%)
Forage	<i>F</i>	80.4	10.4	10.4
Forage	<i>C</i>	78.2	8.9	6.1
Concentrate	<i>C</i>	89.8	11.8	17.4

related bacterial phyla. Regions of ambiguous alignment were excluded from the analysis, and the final alignment was limited to the length of the cloned sequences. Phylogenetic trees were constructed using the PHYML v2.4.5 maximum likelihood analysis program (Guindon and Gascuel 2003). The general time reversible nucleotide substitution model (Tavare 1986) was used with estimated substitution frequency and rate distribution parameters. Nonparametric bootstrap analysis was performed ( $n = 100$ ) to determine node support, and nodes with values greater than 50% were considered well-supported.

### Statistical analysis

Microbial diversity, defined by richness and evenness of TRFs, was measured by applying Simpson's index (Begon *et al.* 2006) to T-RFLP data. Comparing the TRF profile at each sampling point for a horse to that horse's average profile on that diet with Bray-Curtis metrics (Legendre and Legendre 1998) was used as a measure of microbial consistency. Individual TRF values were analysed using repeated measures with horse, diet and period included as independent variables. Culture based results, microbial diversity, consistency values and faecal pH were analysed by one-way ANOVA for repeated measures using the general linear model procedure in (SPSS)<sup>11</sup>. Significance threshold was set at  $P < 0.05$ .

### Results

All horses had daily forage leftovers on both diets (15% on *diet C* and 30% on *diet F*) resulting in a forage:concentrate ratio on *diet C* of 46:54.

#### Faecal pH

Faecal pH measured on the final day from each period was not affected by diet ( $P = 0.55$ ). Mean  $\pm$  s.d. faecal pH was  $6.63 \pm 0.23$  on *diet C* and  $6.79 \pm 0.27$  on *diet F*.

#### Cultured bacteria

The inclusion of concentrate resulted in a mean 10-fold increase ( $P < 0.01$ ) in LAB as indicated by counts on Rogosa agar (Fig 1). Motile swarming bacteria were also observed on Rogosa plates from all horses when on *diet C* and not at all on *diet F*. There was no effect of diet on culturable enterobacteria as indicated by counts on VRBG agar; however, there were large variations between horses on both diets with counts ranging from log 2.5–5.3 colony forming units (cfu)/g.

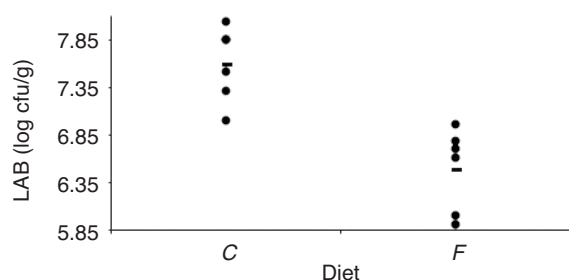


Fig 1: Individual (dots) and mean (dashes) colony forming units (cfu) of lactic acid bacteria (LAB) on Rogosa agar from faecal samples of horses fed a high-energy forage-only diet (F) or a forage-concentrate diet (C). Treatment means were significantly different ( $P < 0.05$ ).

Forty 16S rRNA gene sequences of sufficient quality (48 submitted) were obtained from isolates picked from Rogosa agar. All sequences had matches ( $>99\%$  identity) to previously cultured organisms in RDP. The motile swarming bacteria found only from horses on *diet C* were identified as *Lactobacillus ruminis* (TRF246) in all cases (11 isolates). When they were present, these bacteria swarmed over a large part of the plate (Fig 2). Five isolates corresponded to *L. salivarius* (TRF276), 5 to *L. equi* (TRF276), 3 to *L. mucosae* (TRF67), one to *L. agilis* (M58803) (TRF 276), and 15 to *Streptococcus bovis/equinus* (TRF308). Of the 43 quality sequences from VRBG isolates, 39 were identified as *E. coli* and the other 4 were identified as *E. fergusonii*.

#### Diversity and stability of bacterial composition

From the 6 horses studied a total of 138 TRFs, representing different ribotypes, were found. Microbial diversity, as measured by TRF richness and evenness, was unaffected by treatment ( $P = 0.35$ ) with values mean  $\pm$  s.e.  $0.943 \pm 0.012$  and  $0.948 \pm 0.006$  for *diets C* and *F*, respectively. However, one horse had consistently lower diversity ( $P < 0.02$ ) regardless of diet.

Consistency of the microbial composition within an individual on a diet were generally higher than 60% with an average of 72.6%. The lowest similarity score for each horse on a given diet was consistently lower ( $P < 0.03$ ) on *diet C* than on *diet F*, with mean  $\pm$  s.e. values  $0.640 \pm 0.037$  and  $0.693 \pm 0.021$ , respectively. There was also an affect of horse on stability of the microbial population ( $P < 0.02$ ). Cluster analysis of TRF profiles (Fig 3) shows that samples tended to group according to diet, as well as within horse.

#### TRFs discriminating between diets

Analysis of T-RFLP data identified both TRFs that were more and less abundant on diet C as compared to diet F (Fig 4). TRF276 which corresponded to cultured lactobacilli including *L. salivarius*, *L. equi* and *L. agilis* was more abundant ( $P < 0.05$ ) in *diet C*, but represented only approximately 1% of 16S rRNA genes. All *Streptococcus bovis/equinus* isolates corresponded to TRF308, which was much more abundant ( $P < 0.04$ ) on *diet C* representing an average of 4% of 16S rRNA genes on *diet C* and 0.6% on *diet F*. Other dominant TRFs that were more abundant ( $P < 0.05$ ) on *diet C* included TRF236 and TRF270. Conversely, TRFs that were more abundant ( $P < 0.05$ ) on *diet F* included TRF227 and TRF246. Results of the clone library described below aided in the identification of bacteria corresponding to TRFs indicated above.

#### Clone library

A total of 67 quality sequences (37 *diet F* and 30 *diet C*) were included to build the phylogenetic tree (Fig 5). Clone sequences

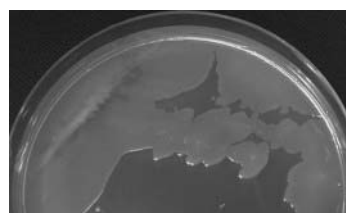


Fig 2: Picture of motile swarming bacteria found only from horses on a diet containing concentrate and identified as *Lactobacillus ruminis* on Rogosa agar.

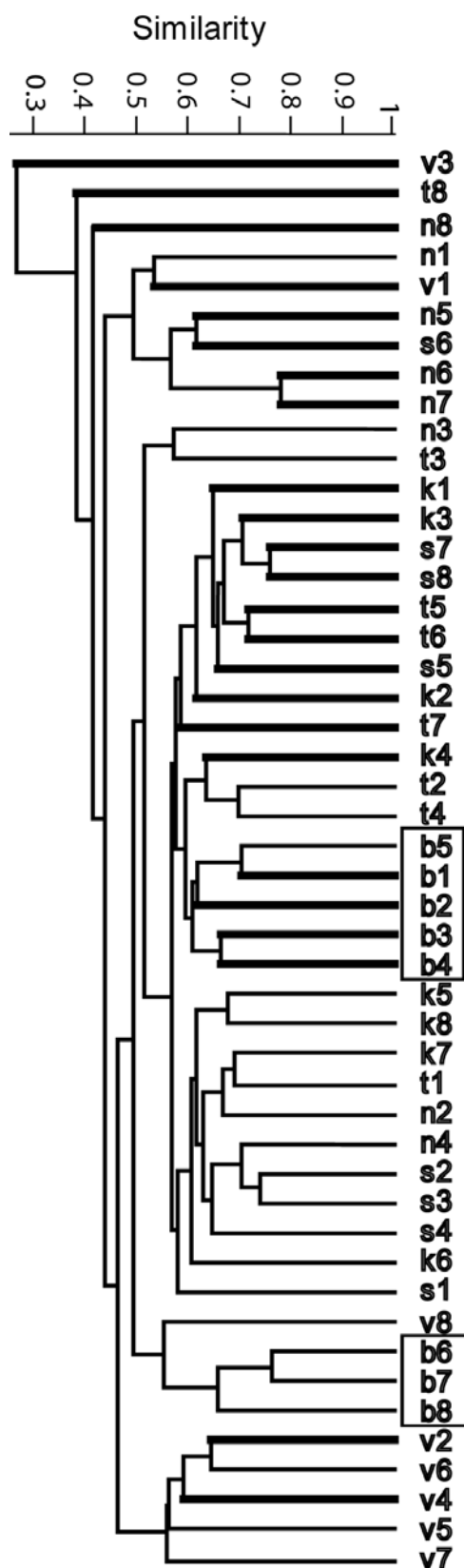


Fig 3: Similarity tree using Bray-Curtis metrics of bacterial 16S rRNA terminal restriction fragment length polymorphism profiles from faecal samples collected from horses on haylage only (narrow lines) or haylage-concentrate (broad lines) diets. Horse is indicated by letter and sampling points are indicated by number in chronological order. Boxes surrounding an individual indicate clustering within horse and diet.

clustered to 3 bacterial divisions; *Firmicutes*, *Bacteroidetes* and *Spirochaetes*. *Bacteroidetes* represented 49% of clones from diet F and only 27% on diet C, while *Firmicutes* represented 73% of clones from diet C and only 46% on diet H. Only 27% of the sequences showed >97% homology to previous database entries. Of the remaining sequences, 57% showed similarity values between 90–97% and 16% showed values between 84–90%. The source of each database entry that gave the best match to the submitted faecal clones were all of faecal origin. Only 15% of the best matches were from the horse, however; 75% were from other hindgut fermenters including other *equids* (zebra and wild ass), elephant, rhinoceros and capybara.

Clones that corresponded to TRFs 236 and 270 were closely related and clustered together among the *Clostridiaceae*, and were all from horses on diet C, in agreement with T-RFLP data. These sequences clustered within *Clostridiaceae* cluster III, although separated from any characterised *Clostridiaceae* suggesting they may form an unknown cluster. All clones that corresponded to TRF246 clustered within the order *Bacteroidales*, although many did not correspond to any previously assigned groups. There were 9 clones, of varying TRF lengths that clustered within *Clostridiaceae* cluster XIVa, of which 7 were from diet F. The single clone that corresponded to TRF227 was affiliated with the *Spirochaetaceae* and was from a horse on diet F, consistent with the increase in TRF227 on this diet.

### Discussion

The current study is the first to characterise changes in the uncultured bacterial population resulting from a more natural high-energy forage-only diet and a high concentrate diet. Diet F showed lower counts and relative abundance of specific bacterial populations that have been associated with the induction of laminitis. By using molecular techniques, it was possible to identify previously unreported changes in bacterial population that corresponded to the presence or absence of readily fermentable carbohydrates in the diet. There was no effect of diet on the diversity of the bacterial population, although the absence of concentrate from the diet resulted in a more stable microbial population. Interestingly, the starch intake level in the current

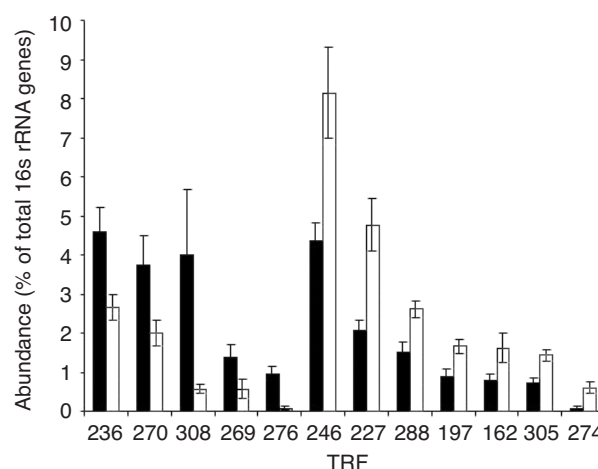


Fig 4: Relative abundance of terminal restriction fragments (TRFs) representing different ribotypes that were significantly more abundant in the faecal microbiota of horses fed a forage-concentrate (C) diet or more abundant on a high-energy forage-only (F) diet. All TRFs indicated were significantly different ( $P < 0.05$ ) between diets. ■ = C; □ = F.

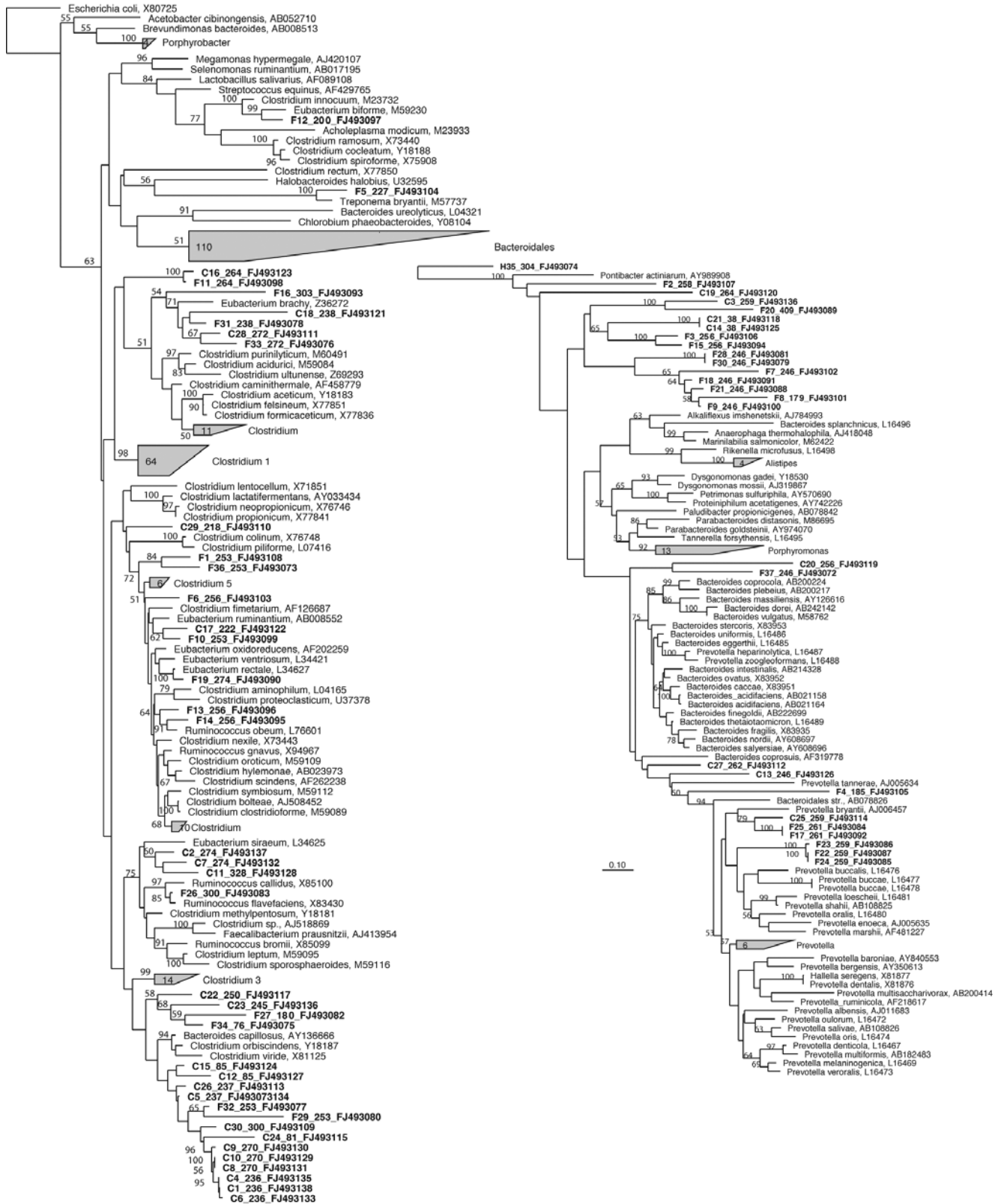


Fig 5: Maximum likelihood tree of 16S rRNA sequences, with *E. coli* as an outgroup. Branch length indicates the nucleotide substitution rate, and node values show percent support as determined by nonparametric bootstrapping ( $n = 100$ ). Values below 50% are not shown. Polygons indicate groups of sequences that are not shown due to spatial constraints within the page, with the number of grouped sequences indicated inside the polygon. Cloned sequences from horses on a high-energy forage only diet are identified with an F, and those from horses on a forage-concentrate diet are identified with a C. Terminal restriction fragment length and accession number are also indicated for each clone.

study is considered to be a safe level of dietary starch inclusion in horses with respect to digestive disturbances (Kienzle 1994; Julliard *et al.* 2008). This merits further studies at both physiological and microbial levels, as it indicates that current feeding recommendations for safe starch inclusion levels in the diet of horses may have to be re-evaluated and possibly changed.

The increased counts and relative abundance of lactobacillar and streptococcal species when concentrate was fed coincides with the findings of several other *in vitro* and *in vivo* studies, where readily fermentable carbohydrates were supplemented (Kern *et al.* 1973; Goodson *et al.* 1988; de Fombelle *et al.* 2003; Varloud *et al.* 2007). In other studies, where fructooligosaccharides, OF or inulin have been supplemented, a reduction in faecal pH has been observed as a result of increased lactic acid production by LAB (Berg *et al.* 2005; Milinovich *et al.* 2006; Crawford *et al.* 2007). Although mean faecal pH was numerically lower on *diet C* in the present study, a significant reduction in pH was not observed. Faecal pH has previously been shown to be a reasonable indicator of caecal and proximal colon pH (Kern *et al.* 1973; Berg *et al.* 2005); however, early and/or subtle changes in caecal pH may have been missed. Repeated measurements over the course of the day may have revealed differences between diets as fluctuations in pH within a horse occur over the course of the day (Goodson *et al.* 1988).

The observation of *L. equi*, *L. mucosae*, *L. agilis* and *L. salivarius* is consistent with *Lactobacillus* species cultured previously from the horse (Yuki *et al.* 2000; Morotomi *et al.* 2002; Al Jassim *et al.* 2005; Endo *et al.* 2007). To the contrary, the motile swarming bacteria present in all horses while on *diet C* and not at all in *diet F*, identified as *L. ruminis*, have not previously been reported in the horse. Since these bacteria were swarming over a large part of the agar surface, many other LAB were probably overgrown which may have resulted in an underestimation of the number of LAB and detection of other species. Although *L. ruminis* and some other lactobacilli have been reported to be motile (Reuter 2001; Rodas *et al.* 2006; Chao *et al.* 2008), the isolates described in this study are, to the authors' knowledge, the first example of swarming lactobacilli. The source of *L. ruminis* may have been the concentrate feed; however, the fact that it was dominant within the culturable LAB on *diet C* indicates that it was colonising and not simply a transient organism.

In a recent report by Milinovich *et al.* (2008), on the changes in hindgut bacteria in OF-induced laminitis, it was suggested that laminitis results from the proliferation and subsequent death *en masse* of streptococcal species. Consistent with this hypothesis, it was observed in the present study that a substantial increase occurred in the relative abundance of *Streptococcus bovis/equinus* on *diet C* and large fluctuations of this population occurred between sampling points in 2 of the horses (data not shown). There was, however, no clinical manifestation of laminitis. The consistently low relative abundance of *Streptococcus bovis/equinus* on *diet F* may support the use of such a diet in trying to prevent gastrointestinal disorders in the horse.

Although there has been a substantial increase in the amount of 16S rRNA gene sequences from gut bacteria, there is still a lack of information in the horse. This is evidenced by the fact that only 27% of sequences found in this study had database matches above 97%, which is only slightly better than the 11% observed in a study performed previously (Daly *et al.* 2001). Furthermore, the source of the majority of the nearest matches was not the horse. Interestingly, most of the nearest matches were from clone libraries obtained from animals that rely on hindgut fermentation as the

main source of energy harvest. This is consistent with the finding that host diet and phylogeny are important in shaping the composition of the intestinal microbiota (Ley *et al.* 2008).

The large representation of clones from *diet F* clustering with members of *Clostridiaceae* cluster XIVa is consistent with a previous report of pasture fed horses (Daly *et al.* 2001). The much lower frequency of clones from *diet C* in this cluster may be associated with lower butyrate production, as members of this cluster are largely responsible for butyrate production in the human colon (Barcenilla *et al.* 2000). Interestingly, the administration of short-chain fructooligosaccharides, which prevented some of the microbial changes associated with carbohydrate overload also resulted in increased butyrate production (Respondek *et al.* 2008). The impact of the specific increase in the *Clostridiaceae* cluster III on the concentrate diet is unknown. Shifts in the representation of *Clostridiaceae* resulting from readily fermentable carbohydrate inclusion have not previously been reported in the horse; however, the shift observed here may correspond to the dramatic shift toward *Clostridiaceae* cluster IX in ruminants switched from high fibre to high grain diets (Tajima *et al.* 2000). As there are no closely related cultured organisms to the observed *Clostridiaceae* cluster, characteristics of bacteria in this group including tolerances and fermentation profiles can only be guessed. One might expect that they are either particularly competitive in utilising readily fermentable carbohydrates or are tolerant to increased lactic acid production resulting from the proliferation of LAB.

Sequence information and T-RFLP data also indicated that the inclusion of readily fermentable carbohydrates into the diet resulted in a reduced representation of *Bacteroidetes* with a specific diminution of an unknown *Bacteroidales* cluster. This agrees with a shift from a predominantly Gram-negative to Gram-positive population upon OF administration (Milinovich *et al.* 2006).

Some general observations regarding the microbiota of the horse could also be inferred from this experiment. Although it was clear that diet had a large effect on microbial composition, the individual horse had a significant effect on microbial diversity and stability. Samples from the same horse tended to cluster together suggesting that some horses may be more or less susceptible to dietary change.

This study indicates that a forage-only diet induces greater microbial stability, while reducing representation of members associated with gastrointestinal disorders, and also that inclusion of high-energy forage may be a viable means to improve animal health in performance horses. There continues to be a lack of knowledge surrounding the uncultured microbiota of the horse. Further investigation into the properties of bacterial groups that appear and disappear with changes in diet is warranted and may provide new insights into ways to improve the feeding and gastrointestinal health of the horse.

#### Manufacturers' addresses

- <sup>1</sup>Miner Röd, Krafft, Sweden.
- <sup>2</sup>Radiometer Denmark, Copenhagen, Denmark.
- <sup>3</sup>Wolf Laboratories, Pocklington, York, UK.
- <sup>4</sup>Merck, Darmstadt, Germany.
- <sup>5</sup>Oxoid, Basingstoke, Hampshire, UK.
- <sup>6</sup>Qiagen, Hilden, Germany.
- <sup>7</sup>MP Biomedicals, Solon, Ohio, USA.
- <sup>8</sup>New England BioLabs, Ipswich, Massachusetts, USA.
- <sup>9</sup>Applied Biosystems, Foster City, California, USA.
- <sup>10</sup>Invitrogen, Carlsbad, California, USA.
- <sup>11</sup>SPSS, Chicago, Illinois, USA.

## References

- Al Jassim, R.A., Scott, P.T., Trebbin, A.L., Trott, D. and Pollitt, C.C. (2005) The genetic diversity of lactic acid producing bacteria in the equine gastrointestinal tract. *FEMS Microbiol. Lett.* **248**, 75-81.
- Bailey, S.R., Marr, C.M. and Elliott, J. (2004) Current research and theories on the pathogenesis of acute laminitis in the horse. *Vet. J.* **167**, 129-142.
- Barcenilla, A., Pryde, S.E., Martin, J.C., Duncan, S.H., Stewart, C.S., Henderson, C. and Flint, H.J. (2000) Phylogenetic relationships of butyrate-producing bacteria from the human gut. *Appl. Environ. Microbiol.* **66**, 1654-1661.
- Begon, M., Harper, J. and Townsend, C. (2006) *Ecology: From Individuals to Ecosystems*, 4th edn., Blackwell, Oxford. pp 471-472.
- Berg, E.L., Fu, C.J., Porter, J.H. and Kerley, M.S. (2005) Fructooligosaccharide supplementation in the yearling horse: Effects on faecal pH, microbial content, and volatile fatty acid concentrations. *J. Anim. Sci.* **83**, 1549-1553.
- Chao, S.H., Tomii, Y., Sasamoto, M., Fujimoto, J., Tsai, Y.C. and Watanabe, K. (2008) *Lactobacillus capillatus* sp. Nov., a motile bacterium isolated from stinky tofu brine. *Int. J. Syst. Evol. Microbiol.* **58**, 2555-2559.
- Clarke, L.L., Roberts, M.C. and Argenzio, R.A. (1990) Feeding and digestive problems in horses - physiological-responses to a concentrated meal. *Vet. Clin. N. Am.: Equine Pract.* **6**, 433-450.
- Cole, J.R., Chai, B., Farris, R.J., Wang, Q., Kulam-Syed-Mohideen, A.S., McGarrell, D.M., Bandela, A.M., Cardenas, E., Garrity, G.M. and Tiedje, J.M. (2007) The ribosomal database project (rdp-ii): Introducing myrdp space and quality controlled public data. *Nucleic Acids Res.* **35**, D169-172.
- Crawford, C., Sepulveda, M.F., Elliott, J., Harris, P.A. and Bailey, S.R. (2007) Dietary fructan carbohydrate increases amine production in the equine large intestine: Implications for pasture-associated laminitis. *J. Anim. Sci.* **85**, 2949-2958.
- Daly, K., Stewart, C.S., Flint, H.J. and Shirazi-Beechey, S.P. (2001) Bacterial diversity within the equine large intestine as revealed by molecular analysis of cloned 16S rRNA genes. *FEMS Microbiol. Ecol.* **38**, 141-151.
- de Fombelle, A., Varloud, M., Goachet, A.G., Jacotot, E., Philippeau, C., Drogoul, C. and Julliand, V. (2003) Characterization of the microbial and biochemical profile of the different segments of the digestive tract in horses given two distinct diets. *Anim. Sci.* **77**, 293-304.
- Dicksved, J., Halfvarson, J., Rosenquist, M., Järnerot, G., Tysk, C., Apajalathi, J., Engstrand, L. and Jansson, J.K. (2008) Molecular analysis of the gut microbiota of identical twins with Crohn's disease. *ISME J.* **2**, 716-727.
- Edwards, U., Rogall, T., Blocker, H., Emde, M. and Bottger, E.C. (1989) Isolation and direct complete nucleotide determination of entire genes. Characterization of a gene coding for 16S ribosomal RNA. *Nucleic Acids Res.* **17**, 7843-7853.
- Endo, A., Okada, S. and Morita, H. (2007) Molecular profiling of lactobacillus, streptococcus, and bifidobacterium species in faeces of active racehorses. *J. Gen. Appl. Microbiol.* **53**, 191-200.
- Goodson, J., Tyznik, W.J., Cline, J.H. and Dehority, B.A. (1988) Effects of an abrupt diet change from hay to concentrate on microbial numbers and physical-environment in the cecum of the pony. *Appl. Environ. Microbiol.* **54**, 1946-1950.
- Guindon, S. and Gascuel, O. (2003) A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. *Syst. Biol.* **52**, 696-704.
- Julliand, V., de Fombelle, A., Drogoul, C. and Jacotot, E. (2001) Feeding and microbial disorders in horses: Part 3 - effects of three hay:grain ratios on microbial profile and activities. *J. equine vet. Sci.* **21**, 543-546.
- Julliand, V., Philippeau, C., Goachet, A.G. and Ralston, S. (2008) Physiology of intake and digestion in equine animals. In: *Nutrition of the Exercising Horse, EAAP Publication No. 125*, Eds: M.T. Saastamoinen and W. Martin-Rosset, Wageningen Academic Publishers, Wageningen. pp 53-70.
- Kern, D.L., Slyter, L.L., Weaver, J.M., Leffel, E.C. and Samuelso, G. (1973) Pony cecum vs steer rumen - effect of oats and hay on microbial ecosystem. *J. Anim. Sci.* **37**, 463-469.
- Kienzle, E. (1994) Small intestinal digestion of starch in the horse. *Revue Méd. Vét.* **145**, 199-204.
- Legendre, P. and Legendre, L. (1998) Ecological resemblance. In: *Numerical Ecology*, 2nd English edn., Elsevier Science, Amsterdam. pp 247-302.
- Ley, R.E., Ley, R.E., Hamady, M., Lozupone, C., Turnbaugh, P.J., Ramey, R.R., Bircher, J.S., Schlegel, M.L., Tucker, T.A., Schrenzel, M.D., Knight, R. and Gordon, J.I. (2008) Evolution of mammals and their gut microbes. *Science* **320**, 1647-1651.
- Ludwig, W., Strunk, O., Westram, R., Richter, L., Meier, H., Yadukumar, Buchner, A., Lai, T., Steppi, S., Jobb, G., Förster, W., Brettske, I., Gerber, S., Ginhart, A., Gross, O., Grumann, S., Hermann, S., Jost, R., König, A., Liss, T., Lubmann, R., May, M., Nonhoff, B., Reichel, B., Strehlow, R., Stamatakis, A., Stuckmann, N., Vilbig, A., Lenke, M., Ludwig, T., Bode, A. and Schleifer, K. (2004) ArB: A software environment for sequence data. *Nucleic Acids Res.* **32**, 1363-1371.
- Medina, B., Girard, I.D., Jacotot, E. and Julliand, V. (2002) Effect of a preparation of *Saccharomyces cerevisiae* on microbial profiles and fermentation patterns in the large intestine of horses fed a high fiber or a high starch diet. *J. Anim. Sci.* **80**, 2600-2609.
- Milunovich, G.J., Burrell, P.C., Pollitt, C.C., Klieve, A.V., Blackall, L.L., Ouwkerk, D., Woodland, E. and Trott, D.J. (2008) Microbial ecology of the equine hindgut during oligofructose-induced laminitis. *ISME J.* **2**, 1089-1100.
- Milunovich, G.J., Trott, D.J., Burrell, P.C., Croser, E.L., Al Jassim, R.A., Morton, J.M., van Eps, A.W. and Pollitt, C.C. (2007) Fluorescence *in situ* hybridization analysis of hindgut bacteria associated with the development of equine laminitis. *Environ. Microbiol.* **9**, 2090-2100.
- Milunovich, G.J., Trott, D.J., Burrell, P.C., van Eps, A.W., Thoenfer, M.B., Blackall, L.L., Al Jassim, R.A., Morton, J.M. and Pollitt, C.C. (2006) Changes in equine hindgut bacterial populations during oligofructose-induced laminitis. *Environ. Microbiol.* **8**, 885-898.
- Morotomi, M., Yuki, N., Kado, Y., Kushiro, A., Shimazaki, T., Watanabe, K. and Yuyama, T. (2002) *Lactobacillus equi* sp. nov., a predominant intestinal lactobacillus species of the horse isolated from faeces of healthy horses. *Int. J. Systemat. Evol. Microbiol.* **52**, 211-214.
- Muyzer, G., de Waal, E.C. and Uitterlinden, A.G. (1993) Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. *Appl. Environ. Microbiol.* **59**, 695-700.
- Respondek, F., Goachet, A.G. and Julliand, V. (2008) Effects of dietary short-chain fructooligosaccharides on the intestinal microflora of horses subjected to a sudden change in diet. *J. Anim. Sci.* **86**, 316-323.
- Reuter, G. (2001) The lactobacillus and bifidobacterium microflora of the human intestine: Composition and succession. *Curr. Issues Intest. Microbiol.* **2**, 43-53.
- Rodas, A.M., Chenoll, E., Macian, M.C., Ferrer, S., Pardo, I. and Aznar, R. (2006) *Lactobacillus vini* sp. nov., a wine lactic acid bacterium homofermentative for pentoses. *Int. J. Syst. Evol. Microbiol.* **56**, 513-517.
- Suau, A., Bonnet, R., Sutren, M., Gordon, J.J., Gibson, G.R., Collins, M.D. and Dore, J. (1999) Direct analysis of genes encoding 16S rRNA from complex communities reveals many novel molecular species within the human gut. *Appl. Environ. Microbiol.* **65**, 4799-4807.
- Tajima, K., Arai, S., Ogata, K., Nagamine, T., Matsui, H., Nakamura, M., Aminov, R.I. and Benno, Y. (2000) Rumen bacterial community transition during adaptation to high-grain diet. *Anaerobe* **6**, 273-284.
- Tavare, S. (1986) Some probabilistic and statistical problems on the analysis of DNA sequences. *Lect. Math. Life. Sci.* **17**, 57-86.
- Varloud, M., Fonty, G., Roussel, A., Guyonvarch, A. and Julliand, V. (2007) Postprandial kinetics of some biotic and abiotic characteristics of the gastric ecosystem of horses fed a pelleted concentrate meal. *J. Anim. Sci.* **85**, 2508-2516.
- Wrangell, C.G. (1911) *Handbok för Hästvänner I & II*. (Reprinted 1992) Höök, Sweden.
- Yuki, N., Shimazaki, T., Kushiro, A., Watanabe, K., Uchida, K., Yuyama, T. and Morotomi, M. (2000) Colonization of the stratified squamous epithelium of the nonsecreting area of horse stomach by lactobacilli. *Appl. Environ. Microbiol.* **66**, 5030-5034.

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