Diet related changes in the gastrointestinal microbiota of horses

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Institutionen för husdjurens utfodring och vård **Examensarbete 258**

Swedish University of Agricultural Sciences Uppsala 2008
Department of Animal Nutrition and Management

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Summary

Naturally horses were used to survive on grazing a fibre rich diet and consequently their energy metabolism was dependent on fibre degrading microorganisms inhabiting their gastrointestinal tract. However, modern performance horses are exposed to high intensity training, therefore energy-dense feedstuffs had to be introduced to feed rations to meet their high energy requirements. These changes in the diet resulted also in changes in the microbial ecosystem inhabiting the gastrointestinal tract which may also have consequences for the health of the horse. The aim of this study was to gain a better understanding about diet related changes within microbial populations in the equine gut using both culture based and molecular methods.

Six mature geldings were fed two different diets (haylage-only and haylage-concentrate) in a cross-over design, both periods consisting of 29 days. Faecal samples were used for the culturing work on Rogosa and VRBG plates, for pH measurement and for analysis with T-RFLP. Several bacteria isolated from plates of both diets were identified by sequencing of 16S rRNA.

The switch to a diet comprising concentrate to 50% of DM resulted in a significant increase in abundance of lactobacilli and streptococci, and in the appearance of *Lactobacillus ruminis*, previously not detected in horse. Several other organisms were also significantly affected by the diet as indicated by T-RFLP results. However, identification of some TRFs is inhibited by the lack of knowledge and references in open databases. On the contrary, an effect of diet on numbers of enterobacteria, faecal pH and diversity of the microbial population could not be observed.

The results of this study show that there are diet related changes in horses' gastrointestinal microbiota, indicating that their ecosystem is affected by the diet. Similar patterns could be observed in most of the horses, but also individual differences exist. However, our knowledge is still limited on horses' gastrointestinal microbiology. Therefore further investigations are needed to gain a better understanding of the interactions between gut microbiota and different feeding practices which would help to develop feeding strategies that can support equine health and welfare.

Összefoglalás

Hosszú évezredeken keresztül a ló természetes takarmánya magas rosttartalmú fű-félékből állt, így életfenntartásához rá volt utalva a bélrendszerében megtalálható, rostokat emészteni képes mikroorganizmusokra. A modern sportló energia igénye azonban olyannyira megemelkedett az intenzív tréningek következtében, hogy magas energiatartalmú abraktakarmányok etetése vált szükségessé. Az új takarmányozási szokások pedig változásokat okoztak a ló emésztőrendszerében előforduló mikrobiális populáció összetételében. Ez következményekkel járhat a ló egészségi állapotára nézve is. A jelen kutatás célja e mikrobiális populáció takarmány-függő változásainak feltérképezése volt, mind a klasszikus, mind pedig a molekuláris mikrobiológia eszközeivel.

A kísérlet két 29 napos periódusból állt, hat kifejlett ügető ló takarmányozásával hármas csoportokban, két különböző takarmányadag összetétellel (100% fűszenázs és 50%fűszenázs-50%abrak). Az analízis trágya minták használatával történt, és a pH érték meghatározását, baktérium kolóniák kitenyésztését (Rogosa és VRBG agaron), izolálását és meghatározását, valamint T-RFLP elemzést foglalt magába.

Az abrak felvétele a takarmányadagba a *Lactobacillus* és *Streptococcus* családba tartozó baktériumok felszaporodását okozta, valamint a *Lactobacillus ruminis* faj megjelenését, amelyet lovakban korábban még nem írtak le. Több másik mikroorganizmus is szignifikáns különbséggel fordult elő a különböző takarmányadagokon, amint azt a T-RFLP eredmények mutatják. Azonban ezek meghatározása nehézségekbe ütközik, hiszen tudásunk a lovak emésztőrendszerének mikrobiológiáját illetően még mindig korlátozott, így pedig hiányoznak a referenciaként használható izolátumok. Ezekkel ellentétben az *Enterobacteriacea* családba tarozó baktérium kolóniák előfordulásában nem volt szignifikáns különbség. A trágya minták pH értéke és a mikrobiális populáció diverzitása sem mutatott szignifikáns eltérést a különböző takarmány összetételek között.

Ezen dolgozat eredményei azt támasztják alá, hogy a takarmány összetétele hatással van a lovak bélrendszerének mikrobiológiájára, hasonló változásokat idézve elő a mikrobiális populáció összetételében a lovak többségénél. Azonban az individuális különbségekről sem szabad elfeledkeznünk. Mindemellett jelen tudásunk e témában még nagy mértékben korlátozott, így további kutatásokra lesz szükség annak érdekében, hogy jobban megérthessük az összefüggést a különböző takarmányozási szokások és a lovak bélflórájának változásai között. Ez a tudás elősegítheti olyan takarmányozási körülmények kialakítását, amelyek mind a sportlovak teljesítményére, mind pedig egészségére pozitív hatással van.

Introduction

The horse is an herbivorous species that is adapted to survive by grazing a fibre-rich diet. Consequently it is dependent on using structural carbohydrates as a source of energy and therefore relies on the fibre degrading microbial population in the hindgut. To the contrary, the modern performance horse is exposed to high intensity training and races and thus to an energy-dense diet which can meet its increased requirements. Not surprisingly, changes in the diet, especially introduction of high-starch cereal grains to the feed ration, result in changes in the microbial population inhabiting the gastrointestinal (GI) tract (Kern *et al*., 1973; de Fombelle *et al*., 2001; Drogoul *et al*., 2001; Bailey *et al*., 2003a; de Fombelle *et al*., 2003; Berg *et al*., 2005; Varloud *et al*. 2007).

The change in the GI microbial ecosystem may also have consequences for the health of the horse. In fact several health conditions are connected with feeding management encompassing the quality of feedstuffs, feeding frequency and availability of pasture areas within stable management (Frape, 2004).

Besides the wish for a good performing horse, health issues are both economically important in the horse industry and are also a question of animal welfare. Therefore it is essential to develop feeding practices, which can efficiently supply the hard working horse with required energy and are also able to prevent nutrition related diseases by achieving a balance in the GI microbial population.

One of the most common diseases in horses is colic, especially performance horses, which can even lead to death. Acidosis and laminitis are also important health issues concerning race horses. There is evidence that all these diseases can develop as a result of abnormal changes in the intestinal microbiota, which occur as a consequence of improper diets or feeding management (Garner *et al.,* 1977; Clarke *et al.*, 1990; Milinovich *et al.,* 2005). It is therefore essential that we consider the intestinal microbiota when developing feeding strategies for the horse. However, the relationship between diet, the intestinal microbiota and horse health are poorly understood and require further investigation. Gaining a better knowledge about this area could help stable managers and horse owners apply feeding practices, which will lead to a healthy GI microbial ecosystem and consequently support equine health and welfare.

Literature review

Horse Nutrition

Historically

The gastrointestinal tract of the horse is the product of having lived on open pasture for millions of years and being dependant on grazing as the main feed intake and source of energy and nutrients. Naturally their diet was an almost continuous intake of small meals of grass rich in fibre, protein and lipid, but low in starch. Consequently the horses' GI tract is structurally and functionally different from other monogastric animals (Frape, 2004).

The stomach

The stomach is the first example of this adaptation. It is a small organ in the adult horse with a rapid passage of digesta and thus tailored to continuous grazing of small amounts. It contains different regions with different functions and physiological conditions, with the pH ranging from 2.5 to 5.6 with a high fibre low protein diet (Argenzio *et al*., 1974). The upper and less acidic region of the stomach is where fermentation of carbohydrates yielding lactic acid and volatile fatty acids (VFAs) takes place. Further down in the pyloric region pH tends to decrease due to HCl and pepsin secretion, indicating proteolytic activity, the hydrolysation of proteins into amino acids. Although the efficiency of protein digestion is questioned because of the stomach's small size and the rapid emptying (Frape, 2004).

The pH in an empty stomach can be as low as 1.5-2, but rising rapidly following a meal, especially after a meal high in dry matter (DM) due to inefficient mixture of digesta with gastric juices. If a low pH cannot be achieved in the pyloric region of the stomach, abnormal fermentation and gastric malfunction could be expected (Frape, 2004).

The small intestine

The small intestine is the site of active enzymatic digestion and absorption of nutrients, although it is relatively short in the horse with a rapid transit of digesta. Enzymatic hydrolysation of protein continues, and also emulsification and digestion of fat, yielding fatty acids and glycerol occur. Lacking a gall bladder, bile is continuously excreted from the liver as another result of the adaptation to continuous eating. Besides the function in fat emulsification, bile also acts as a buffer to the acidic digesta leaving the stomach and helps to achieve a pH close to neutral (Frape, 2004) before entering the large intestine. Soluble carbohydrates are also efficiently digested in the small intestine due to α-amylase and α-glucosidase enzymatic activity. Amino acids, fatty acids, glycerol and glucose are absorbed as end products of digestion, as also lactic acid and VFAs that are partly produced in the stomach and partly in the small intestine.

The large intestine

Having grass as their basic feedstuff, horses are dependent on the use of chemical energy provided by the structural carbohydrates of plants. Since the enzymes for their degradation are not endogenously produced, the horse depends on microbial fermentation in the large intestine. The enlargement of this part of the GI tract is characteristic to horses. The caecum and the colon together represent as much as 65% of the GI tract's volume (Frape, 2004), facilitating efficient fibre fermentation in the horse.

Fibre digestion

To hydrolyse fibre, microorganisms need a relatively long time, which is supported by the slow passage rate in the large intestine. The considerably high, close to neutral pH of the hindgut also supports the favourable environment for the fibre degrading microbial population. Fermentation of fibre results in the production of short-chain volatile fatty acids, mainly acetate, propionate and butyrate, which are absorbed to the blood flow and are used as a source for ATP production. VFAs represent the main energy source of the grazing horse with the molar proportions of 85:10:3 for acetate, propionate and butyrate in the hindgut (Mackie and Wilkins, 1988).

Feeding for performance

From the time when horses became a part of human history they have had to adapt to a new environment, including different types of work and to new feeding practices. The extremes of this change are seen in the modern performance horse. The conditions of its management are connected with stable housing and small pasture areas, high intensity training and races, and a diet that meets its high energy requirements. Consequently high-starch cereal grain diets are commonly fed to performance horses supplying them with energy and nutrients in addition to that derived from the forage.

Although there is no relevant information provided about feeding practices of performance horses worldwide, a few studies and common knowledge indicate that cereal-based diets represent the main energy source of the modern performance horse. According to Richards *et al.* (2006) an average of 7.3 kg/day concentrate is fed to racing Thoroughbred horses in Australia, while Southwood *et al*. (1993) surveyed an average of 7.8 kg/day and 7.7 kg/day concentrate in the diet of Thoroughbred and Standardbred horses, supplemented with an average of 3.3 kg/day and 4.1 kg/day hay. Another study from New Zealand showed that a considerable number of horses are fed less than 2.5 kg forage per day (Williamson *et al.*, 2007) having concentrate rich in starch as their main source of energy.

Starch digestion

Starch consist of α-1,4-linked D-glucose units which can be utilised as an efficient energy source after bonds between the sugars are hydrolysed. Digestion of starch begins in the mouth by salivary amylases, although with a limited efficiency due to relatively low amylase levels in the saliva. Then it continues in the stomach, the active gastric fermentation yielding VFAs and lactic acid. Starch degradation becomes more intensive in the small intestine by the activity of secreted enzymes such as α-amylase and α-glucosidase yielding high amounts of glucose absorbed to the blood flow, and acting as the most important energy source of horses fed concentrate-based diets (Frape, 2004). However, if the amount of starch fed is more than 0.4% of body weight (Potter *et al*., 1992) the capacity of the small intestine might be overwhelmed resulting in undigested readily fermentable starch escaping to the hindgut. This leads to active bacterial fermentation of high soluble carbohydrates in the large intestine, producing lactic acid and a higher proportion of propionate within VFAs. Consequently a drop in pH follows, creating an altered environment for mainly fibre degrading organisms in the hindgut.

Feed constituents

Concentrate

Although the availability of different feedstuffs in the horse industry is mainly dependent on geographical, economical situations and climate, cereal grains containing a high level of starch are frequently used in diets of performance horses worldwide. Other feed constituents commonly included in the concentrate ration are for example cereal by-products and protein concentrates.

Cereal grains

Cereal grains are energy-dense feedstuffs with high starch and DM content. They contain from approximately 10 MJ to 14 MJ ME/kg DM compared to an average hay of 8 MJ ME/kg

DM (Frape, 2004). For example oats and barley are commonly fed to performance and hobby horses as well throughout the world. Whereas oats are relatively rich in fibre, grain-meals in general provide the horse with a high amount of readily fermentable carbohydrate yielding glucose after enzymatic hydrolysation, resulting in a shift from VFA- to glucose-based energy metabolism. Their crude protein content varies between 80 and 120 g/kg DM (McDonald *et al*., 1988), but their nutritional value is relatively low due to their deficiency in essential amino acids like lysine, threonine and methionine. They contain oil from 15 to 50 g/kg (Frape, 2004) which is rich in polyunsaturated fatty acids. Phosphorus is mainly present in the form of phytate salts and in levels three to five times higher than calcium. Phytates can reduce the rate of absorption of other minerals like calcium, zinc and magnesium, which could increase the risk of calcium deficiency as it is present only in low levels in cereal grains (Frape, 2004).

Cereal by-products

Cereal by-products can originate either from the milling or from the brewing and distilling industries, but probably the most frequently fed cereal by-products are derived from wheat-milling, including the germ, bran, course middling and fine middling. Whereas the germ is usually too expensive for horse feeding, wheat bran is commonly used in feed rations. Wheat bran contains around 85- 110 g crude fibre/kg and 140-160 g crude protein (Frape, 2004). There is approximately the same amount of crude protein present in middlings, but their fibre content is lower than of bran. As these by-products are deficient in calcium, but they contain higher amounts of phosphorus in the form of phytate salts, corrections have to be made to balance the mineral content of the feed ration. On the other hand cereal by-products supplement the horse with some of the water soluble vitamins (Frape, 2004).

Protein concentrates

The most common sources of vegetable protein included in concentrates for horses are oil-seed residues after the extraction of oil, peas and beans. These feedstuffs contain much higher amounts of protein than cereal grains with a composition of amino acids of a higher nutritive value. Their oil content in general is relatively low. Oil-seed meals are usually deficient in calcium, but they contain higher amounts of phosphorus and vitamins of the B group (Frape, 2004).

Especially soy bean oil meal is considered to contain proteins of a very good quality. However, there are antinutritive compounds present in raw soya beans; therefore they have to be heat treated prior to consumption. Their crude protein content is around 440-490 g/kg and the fibre content ranges between 15 and 55 g/kg (Frape, 2004).

Peas contain less protein than soya, but their biological value is also of considerably good quality. Because of their tannin content peas should be cooked before feeding (Frape, 2004).

Beans can be divided into winter and spring varieties with a crude protein content of about 230 g and 270 g/kg, and a crude fibre content ranging between 68 and 78 g/kg. Their protein is of high quality and contains considerable amounts of lysine (Frape, 2004).

Although performance supporting benefits of a concentrate-based diet exist, the importance of calculating feed-rations and feeding management are important to point out, as a number of health issues and gastrointestinal malfunctions are connected with an improper concentrate feeding.

Forage

Being the natural feedstuff for horses, a source of fibre has to be provided throughout the year, even in the winter, when there is no vegetation in continental climate areas. This is the reason why grass has to be preserved.

Hay

One way to preserve grasses and legumes is drying to a DM content of 80% (Horrocks and Vallentine, 1999). The process results in hay with a high fibre and low protein content due to late cut and long drying times in the sun. Quality issues and nutritive values of hay are important questions as hay represents the basic feedstuff for millions of horses worldwide. The high fibre content extends eating time with a higher level of chewing activity and saliva production contributing to achievement of an acidic environment in the pyloric region of the stomach but a neutral pH in the hindgut (Frape, 2004). It also provides hindgut microbiota with energy, carbon and nitrogen sources supporting gastrointestinal health. However beneficial hay feeding is to horses, the importance of a proper harvest and storage is worth mentioning, as dusty hay affects the respiratory system and mould growing in hay can also contribute to negative health effects.

Haylage

Another way to preserve grasses and legumes is to ensile them. The process is based on microbial lactic acid fermentation under anaerobic conditions carried out by *Lactobacilli* naturally present on grass. Silage usually contains 35-45% DM (Horrocks and Vallentine, 1999) preserved by acidic conditions with a pH of 3.5-4.5 as a result of lactic acid produced by microbial fermentation. Haylage is an intermediate between hay and silage as grass is dried for a short time before ensiling, achieving a higher DM content of 50-60% (Frape, 2004). Crude protein and vitamin losses are less than in hay production and the conditions are less acidic than in silage. The higher nutritive value of haylage compared to hay is also a result of the fact that haylage is usually harvested at an earlier stage of maturity while hay is often made of grass in late cut (Frape, 2004).

Haylage matches the positive nutritive effects of hay and can overcome the problems caused by weather during hay harvesting in more humid countries. However, a limitation of using haylage-diet for horses is the size of the bales if feeding a small number of horses, as an open bale has to be consumed within some days in order to maintain good feed hygiene. Quality issues must be taken into consideration as gastrointestinal diseases as well as mycotoxicosis can be caused by bad quality silage or haylage (Horrocks and Vallentine, 1999).

Horses' gastrointestinal microbiology

Microbial population in the GI tract

A large and diverse microbial ecosystem inhabits the whole GI tract of horses. They are important for efficient digestion, especially fibre degradation and contribute to equine health and welfare.

The stomach

In spite of the acidic environment, there is a considerable amount of bacteria (~10^6 – 10^8 cfu/mL) present in the stomach, mainly *Lactobacilli*, *Streptococci* and lactate-utilising

bacteria (Varloud *et al.*, 2007). *Lactobacillus salivarius*, *L. crispatus*, *L. reuteri* and *L. agilis* were isolated from the non-secreting area of the stomach, and it is also suggested that some strains would be highly host specific, indicating a symbiotic relationship of Lactobacilli with the horse (Yuki *et al*., 2000). *L. mucosae* and *L. delbrueckii* were also identified from the stomach of roughage fed horses (Al Jassim *et al*., 2005).

The small intestine

Strictly anaerobic bacteria colonise the small intestine of the horse, with numbers ranging from 10^6 to 10^9 cfu/mL, mainly representatives of *Lactobacilli*, *Enterobacteria*, *Enterococci*, *Streptococci* and lactate-utilising bacteria. Whereas *Lactobacilli* are dominant inhabitants of the stomach, *Streptococci* seem to dominate in the small intestine (de Fombelle *et al*., 2003). High amounts (10^6-10^7 cfu/mL) of proteolytic bacteria were found in grass-fed horses (Mackie and Wilkins, 1988) and the presence of *Clostridia sp*., *Proteus sp*., *Staphylococci sp*., *Pseudomonas sp*. and *Candida sp*. has also been reported at low levels in the small intestine (Julliand, 2005). According to de Fombelle *et al*. (2003) total anaerobic bacterial concentration tends to be slightly higher in the stomach and small intestine than in the hindgut of horses fed a concentrate based diet, while they are more homogeneous in the GI tract of forage-fed horses.

The large intestine

The large intestine offers a favourable environment for microbes as pH is around neutral and passage rate is slow. Consequently the microbial population of the hindgut is diverse and is present in as high numbers as 10^9 in the caecum and 10^8 in the colon (Mackie and Wilkins, 1988). Concentration of fungal zoospores is around 10 to 10^4 /mL in the caecal content (Julliand, 2005) and 10^3 to 10^5 /mL of protozoa is also described in the content of the caecum and colon with large individual variations (Kern *et al.*, 1973; Moore and Dehority, 1993).

Cellulolytic bacterial numbers vary from 10^4 to 10^7 /mL of intestinal content (Kern *et al*., 1973; Julliand *et al*., 2001) with a higher abundance in the caecum than in the colon, indicating that the caecum is probably the main site of fibre digestion. Julliand *et al*. (1999) identified *Ruminococcus flavefaciens*, *Ruminococcus albus* and *Fibrobacter succinogenes* as the three main cellulolytic bacterial species in the equine caecum, while Daly *et al*. (2001) described *Clostridium spp*., *Ruminococcus spp*., *Butyrivibrio spp*. and *Eubacterium spp*. as the most important cellulolytic and fibrolytic organisms.

Streptococci and *Lactobacilli* are considered to be the main glycolytic and amylolytic bacteria within the hindgut. *Streptococcus bovis* and *S. equinus* (Julliand, 2005), as well as *Lactobacillus salivarius*, *L. mucosae*, *L. delbrueckii* and *Mitsuokella jalaludinii* (Al Jassim *et al.*, 2005) were reported to be dominant lactic acid producing, while *Veillonella sp*. and *Megashpera sp*. are the main lactate-utilising inhabitants of the hindgut (Julliand, 2005).

A relatively high number of proteolytic bacteria (10^8 bacteria/g) have been found (Kern *et al*. 1973, Mackie and Wilkins, 1988) in the caecum, which would suggest that there is a substantial availability of protein for microbial degradation.

A study using molecular tools identified low %G+C Gram-positive bacteria and *Cytophaga-Flexibacter-Bacteroides* as the two main groups, and *Spirochaetacea*, *Verrumicrobiales*, high %G+C Gram-positive bacteria and *Proteobacteria* as the minor groups present in the equine large intestine. Interestingly only 5% of all the isolated strains corresponded to already known organisms with available sequences in public databases, indicating the lack of detailed

knowledge in horse intestinal microbiology and suggesting a possible existence of "equine only" groups (Daly *et al*., 2001).

Microbial fermentation

As stated above the horse is highly dependent on microbial fermentation in its energy metabolism, especially by degradation of fibre, as the necessary enzymes for that are not present in any of the mammals. Consequently cellulolytic and fibrolytic bacteria contribute the most to energy utilisation in the forage fed horse by degradation of structural carbohydrates in the hindgut. This process results in production of VFAs, mainly acetate, propionate and butyrate in proportions of 85:10:3 (Mackie and Wilkins, 1988) which are utilised as energy source by the horse.

Readily fermentable carbohydrates like starch are efficiently digested by the horses own enzymes yielding glucose as an energy source. However, starch is also efficiently utilised by several bacteria in the GI tract, mainly by lactic acid producing bacteria such as *Streptococci* and *Lactobacilli* (Varloud *et al*., 2007). This results in production of high amounts of lactate throughout the GI tract and VFA production with a higher proportion of propionate (Hintz *et al,* 1971).

Due to proteolytic activity of the intestinal microflora and degradation of bacterial protein horses might have an extra source contributing to their amino-acid metabolism. The importance of this amino-acid source is questioned though, as unlike in ruminants the main site of microbial fermentation takes place in the hindgut, behind the small intestine where major nutrients are absorbed.

During their metabolism microbes also produce vitamins of the B-group which can supply the horse with a basic level of these vitamins.

Besides the metabolites which can be utilised to the benefit of the horse, bacteria are also able to produce vasoactive amines by the decarboxylation of amino acids, which are believed to play a role in the onset of acute laminitis (Bailey *et al.,* 2003a; Elliot and Bailey, 2006). Fifteen different amines were identified from equine caecal content with a significantly higher concentration when inoculated with excess starch *in vitro* (Bailey *et al.,* 2003b). It was also shown that *Streptococcus bovis* and several lactobacilli species, including *L. mucosae,* identified from the caecal content have a decarboxylative activity and are able to produce vasoactive amines in the horse (Bailey *et al.,* 2003a).

Effects of diet on microbial ecosystem

As the feed-intake of a horse is the main nutrient source of gastrointestinal microbiota, diet has a large effect on their ecosystem and with that on the gastrointestinal health of the horse. Not surprisingly several studies reported a change in microbial population when horses were fed different diets (Kern *et al*., 1973; de Fombelle *et al*., 2001; Drogoul *et al*., 2001; Bailey *et al*., 2003a; de Fombelle *et al*., 2003; Berg *et al*., 2005; Varloud *et al*. 2007).

Feeding a high-starch diet the microbial ecosystem of the stomach is highly affected, as numbers of the total anaerobic population, especially *Lactobacilli* and consequently lactic

acid and VFA concentration increase. This indicates a high starch degrading activity in the stomach (de Fombelle *et al.,* 2003; Varloud *et al.,* 2007).

Similar changes seem to occur throughout the whole GI tract following an incorporation of cereal grains (oats or barley) in a hay diet. Numbers of total anaerobic bacteria, *Lactobacilli* and *Streptococci* increased in the caecum and colon of ponies and higher concentrations of lactic acid and total VFA were measured with large individual variations (Kern *et al.*, 1973; de Fombelle *et al.*, 2001). These results were also confirmed in an *in vitro* model of carbohydrate overload, extended by a drop in caecal fluid pH as a result of starch or inulin addition (Bailey *et al.*, 2003a). The increased total VFA concentration was also noticed in the faeces of horses supplemented fructooligosaccharide (FOS), followed by a drop in faecal pH (Berg *et al.*, 2005).

Although results of changes in cellulolytic bacterial and protozoan numbers on a grain-hay diet compared to an only-forage diet are contradictory (Kern *et al.*, 1973; Goodson *et al.*, 1988; de Fombelle *et al.*, 2001), higher propionate and lower acetate proportions within VFA production and reduced fibre digestibility indicate a modification of the fibrolytic activity of the microbial population (Hintz et al., 1971; Drogoul *et al.*, 2001; de Fombelle *et al.*, 2003).

These findings indicate that a considerable amount of starch is able to escape the small intestine following high-grain meals and results in a modification of hindgut microbial population. Due to their ability to utilise readily fermentable carbohydrates, mainly *Lactobacilli* and *Streptococci* are affected and they proliferate following excessive nutrient source. Consequently lactic acid and VFAs are produced to a high extent, causing a more acidic environment in the hindgut, which could result in impaired fibrolytic activity.

Impact on horse health

As described above intestinal microorganisms produce several metabolites as a result of their fermentation which can be utilised for the horses benefit. However, an improper diet can cause disturbances in their ecosystem and modify fermentation, resulting in the production of metabolites that can cause health problems for the horse including acidosis, laminitis and colic.

Laminitis

Laminitis is a common and painful disease of the hooves in horses with the symptoms of lameness, bounding digital pulses and warm feet, often a consequence of cellular damage or inflammation of the tissues surrounding the pedal bone. This condition can be efficiently induced experimentally by starch or oligofructose administration, indicating the role of nutrition in the onset of laminitis. Although the mechanism is not fully understood, it is hypothesized that GI microflora contribute to the development of laminitis (Elliott and Bailey, 2006).

The effects of carbohydrate overload were studied on the onset of equine laminitis by Milinovich *et al*. (2005) using oligofructose administration to experimentally induce laminitis. A decrease in faecal pH and a change in microbial profile were measured, as the predominantly Gram-negative population of the faeces was replaced by a dominant population of Gram-positive bacteria, representing mainly Gram-positive rods. The majority of these organisms were identified as *Streptococcus spp*. indicating a possible role of these

bacteria in the onset of laminitis by their proliferation following carbohydrate overload causing lactate accumulation in the caecum and potentially metabolic acidosis.

Mungall *et al*. (2001) investigated the bacterial pathogenesis of equine laminitis in an *in vitro* model and suggested the role of rapidly proliferating *Streptococcus* species in the caecum and colon following carbohydrate overload in the onset of laminitis. These species might activate matrix metalloproteinases (MMPs) within the lamellar structure by exotoxin production. The activation of MMPs is considered to lead to the separation of the basement membrane from the epidermal basal cells resulting in the symptoms of laminitis.

Similar results were obtained by Bailey *et al*. (2003a) in a study on caecal bacteria in an *in vitro* model of carbohydrate overload, where they measured an increase in numbers of *Streptococci* and *Lactobacilli* in caecal content of horses following fructan incubation, some of which were able to produce amines by decarboxylation of amino acids. It was concluded that these amines absorbed to the blood flow could induce changes in the circulation of the digit and cause laminitis.

However, further investigation is needed on microbial induced laminitis, as the exact mechanism is not yet understood.

Acidosis

Acidosis is a well known condition in cattle caused by carbohydrate overload of the rumen, but it also can occur in horses due to too high grain feeding practices. It is characterised by elevated blood lactate levels, which can lead to laminitis, circulatory collapse and even death (Garner *et al.,* 1977).

Varloud *et al*. (2007) showed an increase in numbers of *Lactobacilli* and *Streptococci* and in amounts of VFA and lactic acid in the stomach following a concentrate meal, while higher abundance of *Lactobacilli* and *Streptococci* and an increase in VFA and lactic acid production was also measured in the caecum and colon of horses fed high-starch diets (de Fombelle *et al*., 2001; Kern *et al.,* 1973; Bailey *et al*., 2003a; de Fombelle *et al*., 2003). These changes can lead to lactate accumulation in the GI tract if lactate-utilising bacteria are overwhelmed by the high amount of lactic acid produced (Clarke *et al.,* 1990). Consequently higher amounts of lactate are absorbed to the blood flow, causing lactic acidosis (Garner *et al.,* 1977).

The same process is to be expected in the stomach as a result of large amounts of concentrate feeding at a time. As the achievement of a low pH in the pyloric region is likely inhibited by an improper mix of digesta, lactic acid producing bacteria can survive, proliferate and their activity lead to lactate accumulation (Frape, 2004).

Colic

Colic is a general expression of gastrointestinal pain in horses with a wide range of possible causes and risk factors including; weather, feeding practices, management and parasite control. These risk factors were reviewed by Gonçalves *et al*. (2002) based on 12 different epidemiological studies and it was concluded that feeding practices, especially abrupt changes of food represent the most important risk factor for colic. Considering the results of previously mentioned studies it is likely that changes in the microbial ecosystem of the GI tract can contribute to the onset of colic.

As colic is often associated with elevated blood lactate levels, similar changes of the GI microbiota following high-grain diets may contribute to colic as well as to acidosis (Frape, 2004). A more acidic environment in the intestine, caused by high levels of lactic acid and VFAs in the GI tract can also lead to mucosal damage (Clarke *et al.*, 1990).

Another risk factor is also the production of gas due to high fermentative activity of *Lactobacilli*, which has to be released through the rectum. If this is inhibited they accumulate causing colic in the gut. Gas accumulation in the stomach can lead to gastric tympany and even to gastric rapture (Frape, 2004).

Methods of profiling the microbiota

Culture based and molecular methods

Both culture based and molecular methods are frequently used in microbiological studies with different advantages and weaknesses, which were amongst others reviewed by Zoetendal *et al.* (2004), Spiegelman *et al.* (2005) and Tyson *et al.* (2005).

Culture-based methods

Traditionally, culture based methods were used for many years in microbiological studies, giving the basic knowledge about microbial populations. However, the techniques are labour intensive and are only able to provide information about a small fraction of the diverse microbial population of the GI tract. The reason for that lies mainly in the lack of knowledge about individual growth requirements for a wide number of bacteria and the selectivity of media. But microbes might also be sensitive to different compounds or might have a need for specific combination of environmental factors, which conditions are difficult to control in the laboratory. As a high proportion of the bacteria inhabiting the GI tract are strictly anaerobes, it also requires culturing techniques carried out under anaerobic conditions (Tyson *et al.,* 2005; Zoetendal *et al.,* 2004).

On the other hand, by already known growth requirements culture based methods can obtain information about even less abundant inhabitants of the GI tract, as they can be efficiently cultured by the use of selective media.

Molecular methods of profiling

Molecular methods are based on the use of the small-subunit ribosomal RNA, referred to as 16S rRNA, in microbiological profiling. This sequence is highly conserved with a slow rate of mutation, is present in high copy numbers in the prokaryote cell and is required for normal cellular functions. The conserved parts allow the use of 16S rRNA in molecular profiling, while more variable regions are the basis for distinction and identification of different organisms (Spiegelman *et al.,* 2005).

The use of molecular methods in microbiology makes it possible to study microbial ecosystems as a whole, to observe community shifts and to evaluate changes in microbial populations in relation to changes in their environment. Besides that sequencing of 16S rRNA can also be used as a tool for identification of certain strains isolated from the diverse

ecosystem. Therefore it is advantageous to make use of molecular methods in the profiling of GI microbiota (Zoetendal *et al.,* 2004; Spiegelman *et al.,* 2005).

However, limitations and bias of the methods exist, as every different step during the handling of the samples in the laboratory is a possible source of bias and manipulation of the microbial population. But difficulties can also occur in the identification of certain sequenced strains because of the lack of references in open databases (Zoetendal *et al.,* 2004; Spiegelman *et al.,* 2005).

Terminal Restriction Fragment Length Polymorphism (T-RFLP)

This molecular method is based on the detection of 16S rRNA fragments with different length as a result of enzymatic digestion. First DNA has to be harvested from the sample and amplified by using the Polymerase Chain Reaction (PCR) with one of the primers being fluorescently labelled. Then a restriction digest is carried out on the product with an enzyme of a known cutting profile. This results in labelled fragments of different length which can be separated by digital detection equipment. Based on that a profile of different peaks representing different fragment length is created, where the size of a peak indicates the abundance of the fragment within the sample. Peaks representing different organisms can be identified with the help of already known DNA sequences and restriction profiles in open databases (Zoetendal *et al.,* 2004; Spiegelman *et al.,* 2005).

T-RFLP is a suitable tool to evaluate whole microbial ecosystems and to study community shifts with a potentially high throughput. It is a relatively sensitive method, which necessitates only small amounts of sample for efficient analysis and provides results in a comparatively short time (Zoetendal *et al.,* 2004; Spiegelman *et al.,* 2005).

On the other hand, the choice of restriction enzymes might be a limitation, as it is likely that some cutting profiles will still remain in the more conserved regions. It also should be taken into account that because of sensitivity limits of the method, information about less abundant (less than 0.05% of the total community) organisms is lost. Difficulties may also arise in the identification of different peaks representing different groups of bacteria as there is still a lack of information in open databases (Zoetendal *et al.,* 2004; Spiegelman *et al.,* 2005).

Consequently more information and higher accuracy can be obtained by using both culture-based and molecular methods of profiling.

Experimental work

Background

Although colic is a common disease in horses, and especially performance horses throughout the world, and although there is evidence for a disturbed GI microflora leading to colic, there is a lack of knowledge on horses' intestinal microbiology. Interestingly the microbial ecosystem of other farm animals like the pig or cattle is more studied and understood. However, the studies performed on horses indicate an effect of diet on microbial populations in the GI tract, showing the importance of feeding management in horse health and welfare. Being able to understand the interactions between inhabitants of the GI tract and feeding practices, and the importance of these factors in equine health, there is a need for further investigations regarding intestinal microbiology. The aim of this study was to get a better understanding about the microbial ecosystem of horses' GI tract and its relationship to different diets. Using also culture based and molecular methods in profiling, a wider and more accurate picture of the results is expected.

Material and Methods

Animals and management

Six mature Standardbred trotter geldings were used in the experiment, which was carried out in November and December 2007. The age of the horses was 6.5 ± 0.4 years and their initial body weight 515 ± 21 kg. All horses were in race condition and trained regularly on a track throughout the experimental period.

The geldings were fed two different diets (haylage-only and haylage-concentrate) in groups of three horses in a cross-over design, both periods consisting of 29 days. The haylage-only diet (H) comprised an early cut timothy/meadow fescue haylage while the haylage-concentrate diet (C) comprised a late cut timothy/meadow fescue haylage (50 % of dry matter) from the same field and concentrate (50 % of DM) (82% oats, 14% soy bean oil meal, 2.7% wheat bran and 1.4% sugar). In addition, both diets were supplemented daily with 51 ± 2 g of vitamin and mineral supplementation (Miner Röd, Krafft, Sweden) and 36 ± 1 g sodium chloride, and 34 ± 1 g ground chalk to the concentrate diet. The amount of haylage fed ranged between 13 and 17.4 kg/day on diet H and between 6.3 and 8.4 kg/day on diet C according to the size of the horse. The concentrate ration was divided into three equal meals per day and the amount fed was between 6.3 and 8.5 kg/day also depending on the size of the horse. A proximate analysis of the feedstuffs is shown in Table 1. The feed-allowances were calculated to be iso-caloric (116 ± 5 in the haylage-diet and 117 ± 5 MJ ME/day in the concentrate diet) and iso-nitrogenous (1002 ± 45 in the haylage-diet and 1008 ± 45 g digestible crude protein/ day in the concentrate-diet) on a daily basis.

Faecal samples from all of the horses were collected in the morning on days 7, 14, 21 and 30 in each experimental period. The faecal samples from days 7, 14 and 21 were put into plastic bags individually and placed in a -20 ºC freezer directly after collection. Faecal samples from days 30 were immediately transported to the laboratory for measurement of pH and for culturing of *Lactobacilli* and *Enterobacteria*.

		MJ / kg DM	% of DM			
Sample	DM	ME	CP	NDF	Fructane	Starch
	%					
Haylage diet C, period 1	77,6	9,0	6,3	59,9	4,5	
Haylage diet H, period 1	80,7	10,5	10,5	58,4	0,3	
Haylage diet C, period 2	78,6	8,7	5,9	60,0	5,7	
Haylage diet H, period 2	80,1	10,2	10,3	62,5	0,5	
Concentrate, period 1	89,8		17,2	19,6	0,4	36,0
Concentrate, period 2	89,9		17,6	21,1	0,6	35,6

Table 1. Analysis of the haylage and concentrate fed in diets C and H in both periods.

Culturing of *Lactobacilli* and *Enterobacteria*

After arrival to the laboratory, 10 g of each fresh faecal sample was diluted with autoclaved peptone water, containing 0.2% peptone and 0.05% tween and homogenised with a stomacher (Stomacher 400, Seward).

For *Lactobacilli* the homogenised contents were plated on Rogosa agar (Merck) plates in different dilutions from 10^{-4} to 10^{-9} and incubated under anaerobic conditions using BD GasPak EZ Anaerobe Container System at 37 ºC for 24 hours.

For *Enterobacteria* the homogenates were inoculated on Violet Red Bile Glucose agar (Oxoid)) plates in different dilutions of 10-fold to 10^{-4} and incubated at 37 ºC for 24 hours. However, due to the use of too high dilutions at the first sampling occasion *Enterobacteria* had to be re-cultured from frozen faecal samples stored at -70 ºC.

After the incubation time bacterial colonies were counted and a mean value calculated for each horse.

Four colonies of *Lactobacilli* and *Enterobacteria* from each horse in each period were picked to M.R.S. broth (Oxoid) in case of *Lactobacilli* and to Brain heart broth (Merck) in case of *Enterobacteria*, respectively and incubated for 24 hours at 37 ºC. Tubes were centrifuged, supernatant transferred and stored in one ml autoclaved freezing liquid (0.82 g K_2HPO_4, 0.18 g KH_2PO_4, 0.59 g Na-citrate, 0.25 g $MgSO_4$ x 7 H_2O, 172 ml 87% glycerol /l) at -70 ºC until further analysis.

Identification of *Lactobacilli* and *Enterobacteria*

Lactobacilli and *Enterobacteria,* isolated by culture, were identified by amplification and sequencing of 16S rRNA genes. Amplification was carried out in Ready-to-Go PCR tubes (GE healthcare) using an MJ Mini Personal Thermal Cycler (BIO-RAD) in 25 µl reactions containing 0.5 µl bacterial culture, 1 µl forward primer (5'-AGAGTTTGATCCTGGCTC-3'), 1 µl reverse primer (5'-CGGGAACGTATTCACCG-3') and 22.5 µl water with the following cycling conditions: hot start for 5 min at 94 ºC, followed by 30 cycles of denaturation at 94 ºC

for 30 sec, annealing at 49 ºC for 30 sec and extension at 72 ºC for 2 min, and a final extension at 72 ºC for 10 min.

Quality of PCR products was checked on a 1% agarose gel and purified using a QIAquick PCR Purification Kit (Qiagen) according to the manufacturer's instructions. Purified products were sent for DNA sequence analysis.

Measurement of the pH
Five g of each fresh faecal sample collected on day 30 in both periods was diluted in 20 ml water and pH measured with a PHM 92 LAB pH-meter (Radiometer Copenhagen).

The remainder of the faecal samples from day 30 was stored in individual plastic bags at -20 ºC until further analysis with TRFLP.

T-RFLP analysis
To obtain a profile of the faecal microbiota, total extracted DNA was analysed by T-RFLP. DNA was isolated from 250 mg of all 48 samples (six horses and eight sampling days) using the FastDNA SPIN for Soil Kit (MP Biomedicals, LLC., Ohio, Cat# 6560-200) according to the manufacturer's instructions.

Bacterial 16S rRNA genes were specifically amplified with broad-range bacterial primers Bact-8F (5'-AGAGTTTGATCCTGGCTCAG-3'), which was labelled with 6-carboxyfluorescein on the 5'end and reverse primer 926r (5'-CCGTCAATTCCTTTRAGTTT-3'). The PCR was carried out in duplicate in 25 µl reactions containing 2.5 µl 10x PCR buffer, 1 µl BSA, 1 µl dNTP (5 mM), 1 µl of each primer, 0.25 µl *Taq* polymerase (5 U/µl), 1 µl template DNA and 17.25 µl water using a GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA) thermocycler with the following cycling conditions: hot start for 5 min at 94 ºC, followed by 30 cycles of denaturation at 94 ºC for 20 sec, annealing at 55 ºC for 30 sec and extension at 72 ºC for 30 sec, and a final extension at 72 ºC for 7 min. Quality of PCR products was checked on a 1% agarose gel.

The restriction digest was performed on duplicate samples at 37 ºC for 2 h on 25 µl reactions containing 2.5 µl 10x NE Buffer, 6 µl DNA, 0.25 µl HaeIII enzyme and 16.25 µl water.

The digested products were diluted 10-fold with water and analysed for fragment size on an ABI 3730 capillary sequencer. GS ROX-500 size standards (ABI) were included in each well for determination of fluorescently labelled fragments. T-RFLP profiles were processed using Peak Scanner V 1.0 (Applied Biosystems). Relative peak area of terminal restriction fragments (TRFs) corresponding to sizes between 50 and 500 bp were calculated by dividing individual peak area by total peak area within this size constraint. Only peaks above a threshold of 0.5% were included in further analyses.

Statistical Analysis
Microbial diversity was measured by applying Simpson's index of diversity to T-RFLP data, which is defined by evenness and richness of TRFs. TRF data was analysed using repeated measures with horse, diet and period included as independent variables. Consistency within individual was assessed by comparing each sampling point to the mean on that diet, using Manhattan metrics. Culture based results, microbial diversity, microbial consistency and faecal pH were analysed by one-way ANOVA using the general linear model (SPSS).

Results

Measured pH values

The average faecal pH value (mean ± standard deviation) on diet C was 6.63 ± 0.23 and on diet H 6.79 ± 0.27, which was not significantly different. (Figure 1)

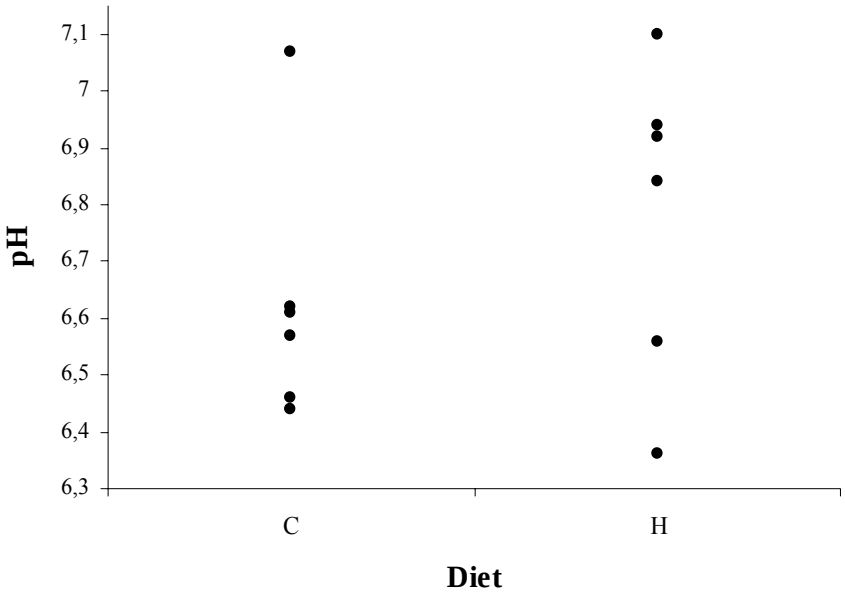

Figure 1. pH values of fresh faecal samples from horses on diets C and H

Bacterial counts on Rogosa plates

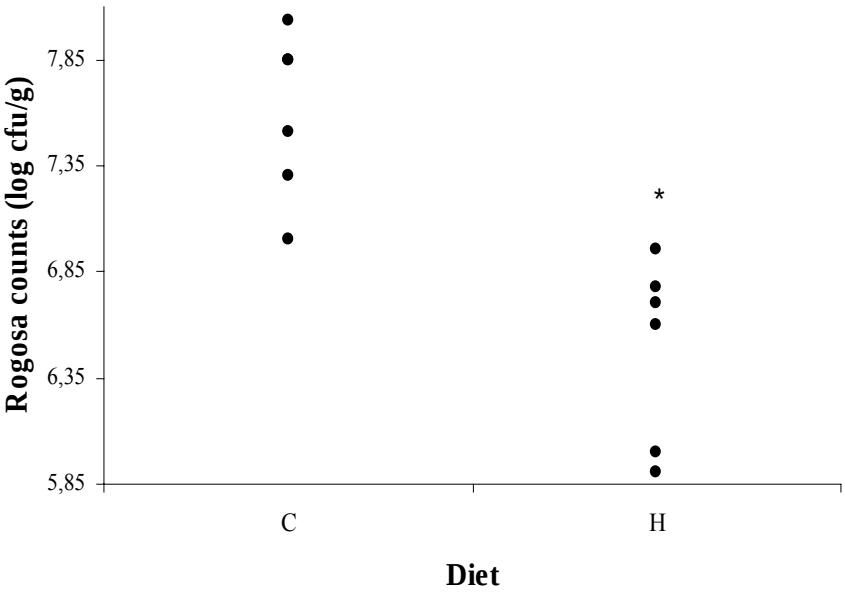

Figure 2. Viable counts on Rogosa plates from fresh faecal samples of horses on diets C and H

Bacterial counts on Rogosa plates were significantly higher ($p<0.05$) on diet C, their numbers ranging from log 7 to log 8.03 cfu/g, and log 5.9 to log 6.95 cfu/g on diets C and H, respectively (Figure 2). Motile swarming bacteria were observed on plates from all horses on diet C, but were absent from all horses when on diet H.

Enterobacterial counts

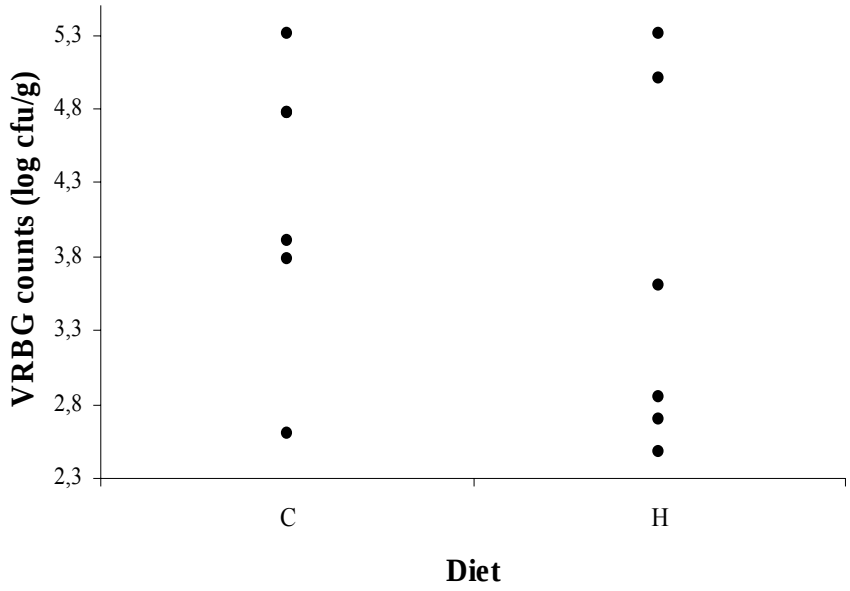

Figure 3. Enterobacterial counts on Violet Red Bile Glucose agar plates from fresh faecal samples of horses on diets C and H

There was no effect of diet observed on enterobacterial counts, as indicated by selective growth on Violet Red Bile Glucose agar. However, large individual variations were noticed between the horses on both diets, the numbers ranging from log 2.5 to 5.3 cfu/g (Figure 3).

Identification of bacteria

16S rRNA gene sequences of sufficient quality were obtained from 40 of 48 isolates (23 of 24 on diet H and 17 of 24 on diet C), picked from Rogosa agar plates. The bacteria were identified using Ribosomal Database Seqmatch and BLAST searches in GenBank. The motile bacteria found only in diet C samples were identified as *Lactobacillus ruminis* in all cases (11 isolates). Five isolates corresponded to *Lactobacillus salivarius*, five to *L. equi*, three to *L. mucosae*, one to *L. agilis*, one to *Streptococcus bovis* and 14 to *S. equinus* (Figure 4).

The types of lactobacilli and streptococci isolated from samples of the two distinct diets differed almost completely. However, it has to be stated that colonies were not picked unbiased, as motile bacteria (identified later as *L. ruminis*) only present on plates from diet C were picked from each sample regardless their abundance. The number of identified bacteria was also relatively low.

Escherichia coli was found to be the dominant culturable enterobacterial species on Violet Red Bile Glucose agar, as except four *Escherichia fergusonii* isolates equally represented on both diets, all the other 39 identified strains matched *E. coli*.

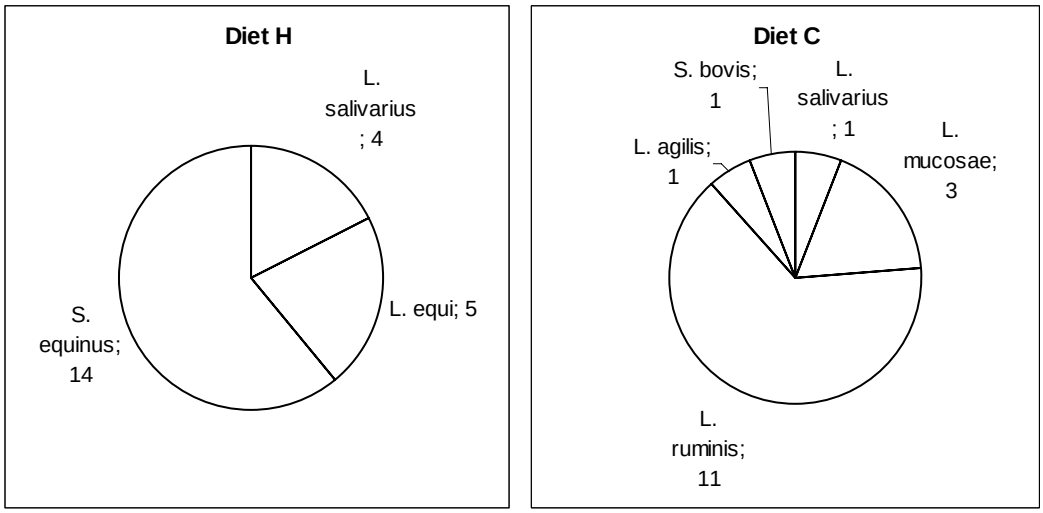

Figure 4. Numbers of lactobacilli and streptococci isolated from horse faecal samples on diets H and C.

T-RFLP results

Diversity

Simpson's diversity indices of the horses, representing a combination of richness and evenness of TRFs within faecal samples, were not significantly different on the diets. With the exception of horse n on diet C, average diversity values ranged from 0.93 to 0.96 on both diets. (Figure 5)

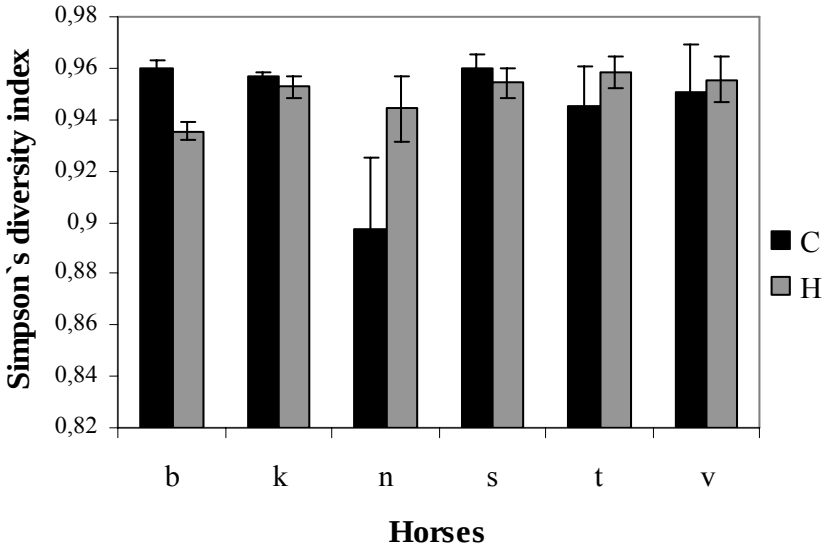

Figure 5. Average Simpson's diversity indices based on TRF profiles of horse faecal samples on diets C and H with error bars representing standard error.

Similarity between samples

The Bray Curtis similarity tree (Figure 6) shows a grouping of samples according to the diets, as diet C samples and diet H samples tend to group together. As within diets it is noteworthy that samples taken from the same horse cluster together. An exception is horse v with samples building two separated groups which do not seem to be related to the diets or to samples of other horses. Average similarity indices of the horses on diet C and H compared to the mean

of the diet ranged between 0.7 and 0.77, with the exception of values from horses n and v ranging between 0.63 and 0.69.

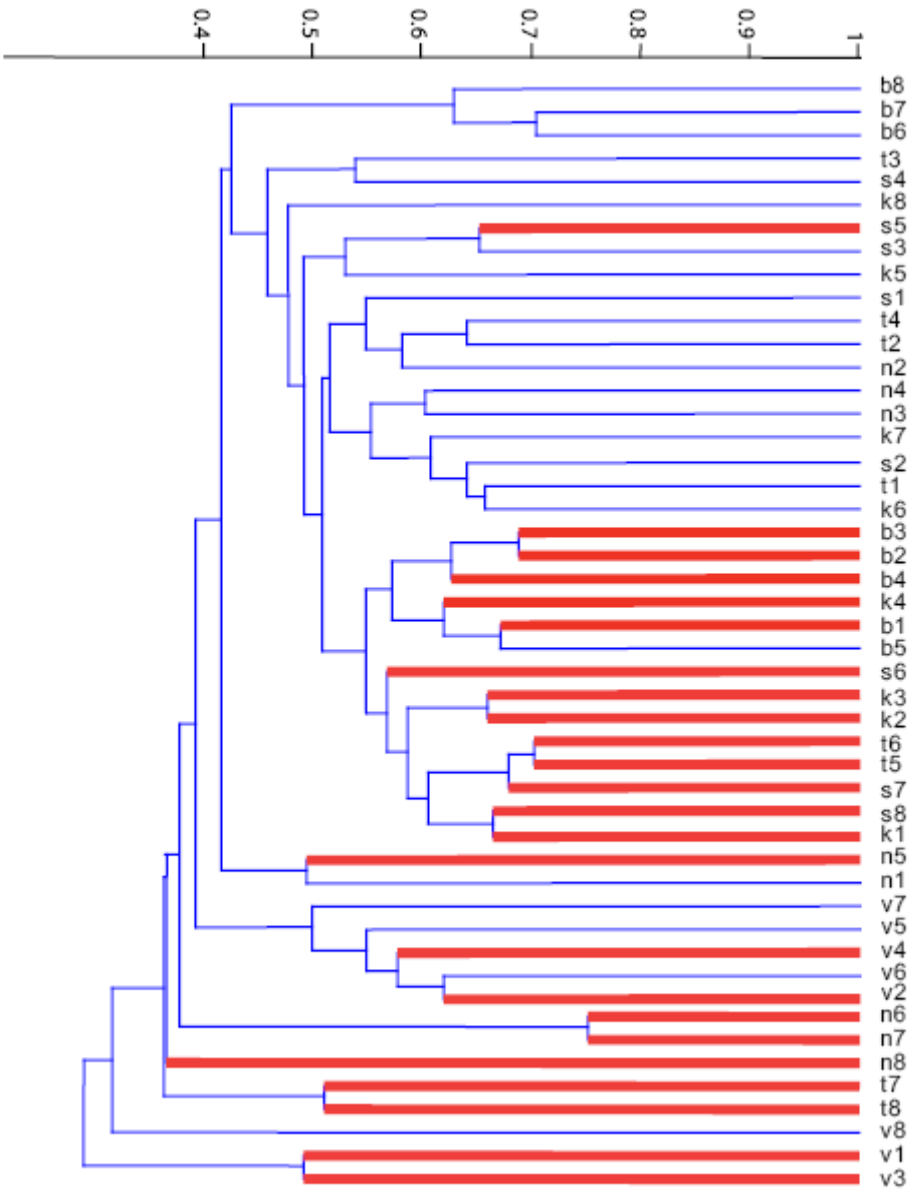

Figure 6. Bray Curtis similarity tree of bacterial 16S rRNA T-RFLP profiles of horse faecal samples on diets C and H. Fat lines indicate samples from diet C and skinny lines samples from diet H. Letters refer to different horses and numbers to the sampling occasions.

Significantly different TRF peaks

T-RFLP analysis of the faecal samples resulted in 156 different TRF peaks with several being significantly different ($p<0.05$) on the diets. The average abundance of peaks 197; 226; 228; 259; 274; 287 and 305 increased on diet H (Figure 7) and 235; 249; 262; 276; 293 and 308 on diet C (Figure 8).

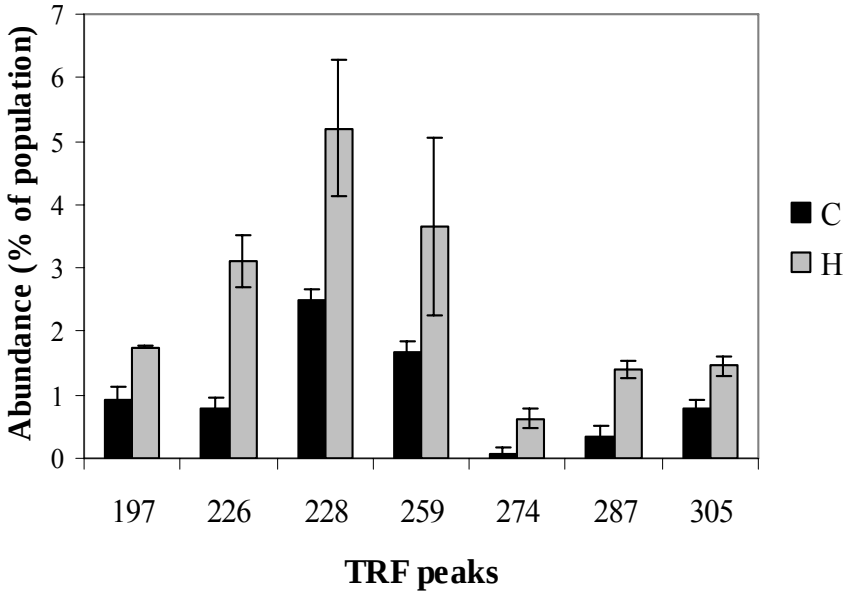

Figure 7. TRF peaks from horse faecal samples with significantly ($p<0.05$) higher abundance on diet H. Error bars indicate standard error.

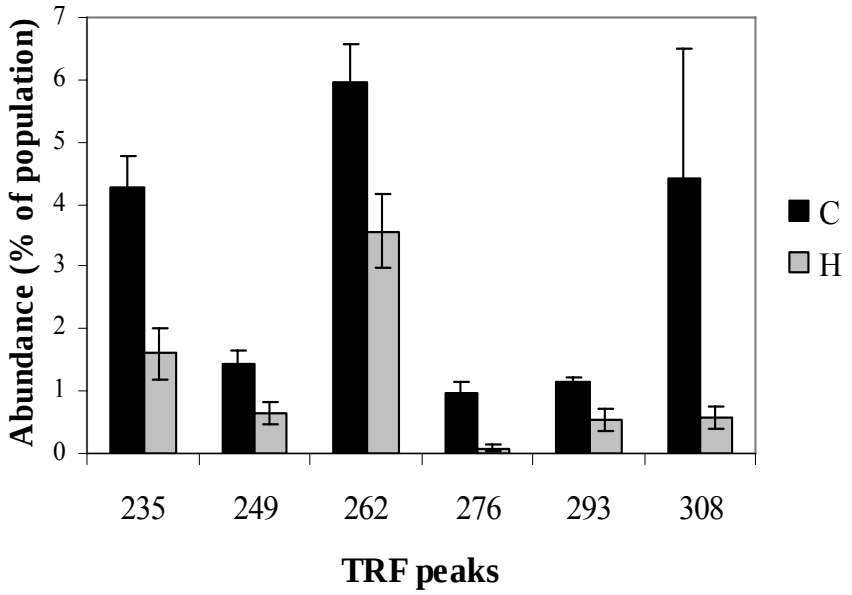

Figure 8. TRF peaks from horse faecal samples with significantly ($p<0.05$) higher abundance on diet C. Error bars indicate standard error.

Restriction sites of *L. salivarius, L. agilis,* and *L. equi* corresponded to peak 276 with a 12-fold increase in abundance on diet C and of *Streptococcus equinus* and *S. bovis* to peak 308 with a 7.5-fold increase also on diet C. However, it is not possible to differentiate between the prevalence of these species within a TRF peak. Based on their cutting profile, *L. ruminis* and *L. mucosae* could be identified as peaks 246 and 67, although these peaks were only present in a very low abundance which in many samples did not even reach the threshold of being taken into further analysis.

Discussion

Although it is generally accepted that changes in the diet result in changes in microbial composition of the GI tract, these changes are poorly understood. By using culture based and molecular based approaches we were able to identify some specific changes in composition that resulted from an introduction of concentrate to the diet. The switch to a diet comprising concentrate to 50% of DM resulted in an increased abundance of lactobacilli and streptococci, and in the appearance of *Lactobacillus ruminis*. Several other groups of micro-organisms were also significantly affected by the diet as indicated by T-RFLP results. However, identification of some TRFs is inhibited by the lack of knowledge and references in open databases. On the contrary, we did not observe an effect of diet on numbers of enterobacteria, faecal pH and diversity of the microbial population.

Effect of diet on lactobacilli and streptococci

Lactobacilli and streptococci were affected by the change of diet, as indicated by both culture based and T-RFLP results. Their numbers increased on average more than ten times when concentrate was fed. Several other studies obtained similar changes in the abundance of lactobacilli and streptococci both *in vitro* and *in vivo* after excess easily fermentable carbohydrate was supplemented (Kern *et al*., 1973; Goodson *et al.*, 1988; de Fombelle *et al*., 2001; Bailey *et al.*, 2003a; de Fombelle *et al.*, 2003; Varloud *et al.*, 2007). This indicates starch availability for microbial metabolism and consequently a proliferation of these bacteria. Although it is not possible to distinguish between the changes in different parts of the GI tract because of the use of faecal samples, the hindgut might also be affected, if higher amounts of starch could escape the small intestine. This would result in excess nutrient source for lactobacilli and streptococci even in the hindgut and consequently in higher production of lactic acid and VFAs. High concentration of VFAs could lead to more acidic conditions in the hindgut and consequently create an altered environment for fibrolytic microbiota.

Some species of lactobacilli were identified from faecal samples in this study, which were all previously reported in horses with the exception of *L. ruminis*. *L. salivarius*, *L. mucosae*, and *L. agilis* were described in different parts of the GI tract (Yuki *et al.*, 2000; Al Jassim *et al.*, 2003), while *L. equi* was isolated from horse faeces (Morotomi *et al*., 2002; Endo *et al*., 2007). On the contrary, *L. ruminis* was found both in cattle (Krause *et al*., 2003) and in pig (Yin and Zheng, 2004), but was not previously described in the horse. This *Lactobacillus* species is a motile, gram-positive rod, which ferments soluble sugars with L-lactate as an end product (Hespell *et al.*, 1997). *L. ruminis* was found in samples of every horse but only on the diet supplemented with concentrate.

Streptococcus bovis and *S. equinus* were also isolated previously from horses and their proliferation following carbohydrate overload was connected with the onset of laminitis. Although the exact mechanism is not fully understood, different hypotheses exist about the contribution of these species to equine laminitis, including activation of MMPs through exotoxin production or the production of vasoactive amines (Mungall *et al.*, 2001; Bailey *et al.*, 2003a; Milinovich *et al.*, 2005; Endo *et al*., 2007). Besides these streptococci, some lactobacilli species including *L. mucosae* and *L. salivarius* were also found to be able of producing amines and therefore might also play a role in the onset of laminitis (Bailey *et al.*, 2003a). Although the threshold for the onset of laminitis is not described, the results of this study show a considerable increase in numbers of streptococci and lactobacilli even on a diet

where concentrate is supplemented to 50% of the DM, featuring a ratio commonly fed to performance horses.

The profiles of isolated streptococcus and lactobacilli strains differed almost completely on the diets, *S. equinus* and *L. equi* being dominant on the haylage, and *L. ruminis* on the concentrate diet. Nevertheless, a reason for the high number of streptococci isolates on diet H might be the very low abundance of lactobacilli on this diet, as it is indicated by T-RFLP results. Therefore it was more likely to pick a *Streptococcus* than a *Lactobacillus* colony from plates of diet H samples. However, these results might not be unbiased, as motile bacteria (identified later as *L. ruminis*) only present on plates from diet C were picked from each sample regardless their abundance. The number of identified bacteria was also relatively low.

Effect of diet on enterobacteria

Concerning numbers of enterobacteria there was no difference found between diets, but there were large individual differences between horses. This on the other hand could be a result of the use of frozen samples in the culturing work of one of the sampling occasions. On both diets *E. coli* was the dominant enterobacterial species, besides the few *E. fergusonii* isolates which were also present on both diets.

Faecal pH

Increased streptococci and lactobacilli numbers in the GI tract are usually indicators of excess readily fermentable carbohydrate and their proliferation connected with higher production of lactic acid and VFAs. These metabolites can result in more acidic conditions in the hindgut, and consequently in a drop of faecal pH, which was reported in three former studies following FOS, oligofructose or inulin supplementation (Berg *et al.*, 2005; Milinovich *et al.*, 2005; Crawford *et al*., 2007). In the contrary, faecal pH was not significantly different on the diets in the present study. Although the average pH value on diet C was numerically lower than on diet H, it also has to be stated that three of the six horses had even a higher pH value on diet C than on diet H. Nevertheless it is noteworthy that the mentioned studies used readily fermentable carbohydrates as a supplementation in high doses, one of which was even enough to induce laminitis, while in the present study oats were used as the main part of the concentrate diet. It is likely that the amount of starch present in oats together with the high fibre content of this cereal grain did not result in too high VFA production and consequently in a drop in faecal pH. However, an increase of faecal pH on the concentrate diet was unexpected.

On the other hand, fluctuations of faecal pH within a horse have to be taken into consideration. Although there is no relevant information on faecal pH variability within a horse, repeated measurements of caecal pH values following a forage or a grain meal showed considerable fluctuations within animals. While a linear decrease followed by a linear increase in caecal pH values were observed in forage fed horses, there was a delay in caecal pH drop post feeding on the grain diet, followed by higher fluctuations within a horse (Goodson *et al.*, 1988). Similar patterns in faecal pH variability might explain the slightly higher pH values measured in this study by some of the horses on the diet supplemented with concentrate.

Diversity

There was no difference noticed between microbial diversity in samples from animals fed different diets. However, it could have been expected that changes in the diet, causing changes in the environment, especially more acidic conditions, would alter the microbial

ecosystem, resulting in a less diverse, less stable population. On the contrary, the results indicate that diet C was not extreme enough to modify the diversity of GI microbial ecosystem. It seems that horses are able to balance their microbiota in case of a diet containing concentrate to 50% of DM and their microbial diversity is not altered. On the other hand, introduction of new feedstuffs to the ration, like concentrate to a haylage-only diet, would also mean a supply of extra nutrients for the microbial population and consequently support the growth of a wider range of organisms. This could have positively affected microbial diversity on the concentrate diet.

Similarity

Similarity results also confirm that the GI microbiota is affected by the diet, as samples tended to group together according to the diets. This indicates that there were several changes in the microbial population with a similar pattern in most of the horses. However, samples also clustered together according to horses, which on the other hand indicated the individuality of microbial populations inhabiting the GI tract of different horses. Especially one of the horses seemed to have an individual microbial ecosystem which was neither related to the diet nor to other horses. This result might emphasize the necessity to consider individualities and special requirements of different horses by developing feed rations and feeding practices.

Changes in TRF profiles

Several TRF peaks showed significant differences on the diets, indicating diet related changes in the microbiota, which had similar patterns in most of the horses. Based on restriction sites of known sequences of lactobacilli and streptococci, isolated in the present study, TRF 176 might be identified as a group of bacteria including *L. salivarius*, *L. agilis* and *L. equi*, and TRF 308 as *S. equinus* and *S. bovis*.

As concerning other TRFs, information in open databases was used for determination. However, the identification of these peaks was difficult, as there is still a lack of knowledge on inhabitants of horses' GI microbial ecosystem. In a study performed 2001, 89% of the sequences isolated from the large intestine of horses did not correspond to already known organisms with available sequences in public databases (Daly *et al*., 2001), and there are still only a few reference strains isolated from the horse GI tract. Consequently it has to be emphasised that the suggestions presented here are only possible identifications of different TRFs, with some organisms only being described in the ruminant, pig or human, but not in the horse. Therefore it might not represent the real picture.

TRF peaks being more abundant on diet H may account for fibrolytic and cellulolytic microorganisms. *Ruminococcus flavefaciens*, *R. albus* and *Fibrobacter succinogenes* were reported previously as the main cellulolytic bacteria in the equine caecum (Julliand *et al*., 1999), of which the two *Ruminococcus* species might be identified as peak 305. Interestingly, restriction sites of other *R. flavefaciens* and *R. albus* strains corresponded to peak 259, while *F. succinogenes* did not correspond to any of the peaks being significantly higher on the haylage-only diet. TRF 274 might be identified as *Butyrivibrio fibrisolvens*, another fibre degrading bacteria of the equine GI tract (Daly *et al.*, 2001). Groups of microorganisms including *Clostridium spp.* (like *Clostridium herbivorans*) and *Eubacterium spp*. (like *Eubacterium ruminantium*) might be represented by TRFs 197 and 287, although bacteria of these groups have not yet been identified on species level in the horse, but are considered to play an important role in fibre degradation (Daly *et al.*, 2001). Unfortunately it is not possible

to suggest potential organisms for TRFs 226 and 228, even though these peaks were relatively abundant on both diets, but especially on diet H.

Regarding peaks with a significant increase in abundance on diet C, certain species of lactobacilli and streptococci can be identified as TRFs 276 and 308, respectively. Different *Prevotella* and *Bacteroides* species might correspond to TRF 262, but identification of TRFs 235, 249 and 293 failed because of the lack of detailed knowledge about horse GI microbiology. The above mentioned groups of bacteria might be able to degrade starch and use it in their metabolism. Consequently they might proliferate on a diet comprising concentrate and possibly replace some populations that dropped significantly following the switch from the haylage-only diet.

However, it also has to be stated that there might have been also other diet related changes in the GI tract of the horses, which might not have been detected because of the use of faecal samples in the analysis.

Conclusion

Results of this study show that there are changes in the microbial ecosystem of the horse following changes in the diet; consequently microbial populations are affected by the differences of diets. Several of these processes have similar patterns in most of the horses, but individual differences should also be taken into consideration. We have also detected an organism previously not found in the horse, *Lactobacillus ruminis*, and observed an increased abundance of lactic acid bacteria when feeding the horses a diet supplemented with concentrate. However, our knowledge on horse GI microbiology is still limited. Therefore further investigations are needed for being able to give a detailed description of the interactions between inhabitants of the GI tract and different feeding practices. Gaining a better understanding about these relations could help in developing feeding strategies that can support equine health and welfare.

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