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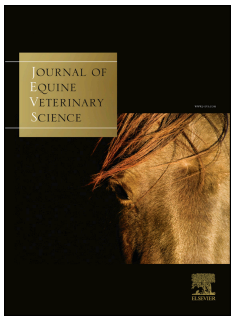

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Effect of live yeast culture supplementation on fibrolytic and saccharolytic bacterial populations in the faeces of horses fed a high-fibre or high-starch diet.

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Short title: Yeast Supplementation and Bacterial Populations in Equine Faeces

Abstract

The objective of this study was to assess the effect of live yeast (*Saccharomyces cerevisiae*) supplementation on the populations of specific cellulolytic (*Fibrobacter succinogenes* and *Ruminococcus flavefaciens*) and saccharolytic (*Streptococcus equinus* and *Streptococcus bovis*) bacteria in the faeces of horses fed high-starch and high-fibre diets. Four horses were each fed diets consisting of high-fibre with no yeast (HF), high-fibre with yeast (HFY), high-starch with no yeast (HS) and high-starch with yeast (HSY) in a 4 × 4 Latin-square design study. Fresh faecal samples were collected on the last 3 days of each 31-day experimental period and were then assessed, using semi-quantitative real-time PCR, for total bacterial load and levels of target bacterial species, relative to the total bacterial load. The most abundant of the target species was *F. succinogenes* and the HSY diet resulted in a significant ($P = 0.045$) reduction in relative levels of this bacterium. No significant effect ($P = 0.224$) of diet was observed in relation to abundance of *R. flavefaciens*. Results show that diet did not have a significant ($P = 0.068$) effect on relative quantities of *S. equinus*, although there appeared to be a trend for increased levels of this bacterium during feeding of high starch diets. Numbers of *S. bovis* were higher ($P < 0.001$) when horses were fed HS and HSY diets than when fed HF and HFY diets. Significant variation in levels of *S. equinus* ($P = 0.024$) and *S. bovis* ($P = 0.049$) was observed between individual horses.

Introduction

Horses have evolved to eat high-fibre diets which are ingested in relatively high volumes over long periods throughout the day. This natural diet is a stark contrast to the high-starch diets (generally considered as those containing over 1g starch per kg bodyweight) frequently fed to performance horses, which often require much more energy than they can gain solely from a fibre-based diet, meaning concentrates form a considerable part of the ration. Such starch-rich diets are known to disrupt the natural environment of the hindgut (for example, increasing numbers of amlolytic bacteria, leading to a decrease in pH) [1], compared to a high-fibre diet, and can result in the development of metabolic disorders, such as hindgut acidosis and laminitis [1-3]. Undoubtedly, the microbial ecology of the equine hindgut is of great importance and a sound knowledge of its function will help in the prevention of disease. However, whilst much work has been done to improve knowledge of the microbial ecology of the equine digestive tract there is still less information available for the hindgut of horses compared with, for example, the colon of pigs [4, 5] and the rumen of cattle and sheep [6, 7].

Moreover, there is a need for an approach to feeding performance horses which provides the required nutrients without detriment to the hindgut. The addition of probiotics, including the yeast Saccharomyces cerevisiae, is one such approach which has been shown to enhance both nutrient digestibility [8, 9] and activity of cellulolytic bacteria, such as Ruminococcus flavefaciens, in the hindgut [10]. However, little attempt has been made to measure the effect of S. cerevisiae on numbers of saccharolytic bacteria present in the hindgut using modern molecular methods. Increased numbers of saccharolytic bacterial species, such as Streptococcus equinus and Streptococcus bovis have been associated with the onset of gastrointestinal problems in the horse, which are often linked to high-starch diets [11]. Thus, the ability to reduce the numbers of such bacteria in the hindgut of horses fed high-starch diets would be valuable to the maintenance of gut health. Supplementation with S. cerevisiae

might be a viable method of achieving this goal of preventing damage to the gastrointestinal tract by altering the bacterial populations present in the hindgut.

This study investigated the effects of yeast supplementation on some major populations of cellulolytic (*R. flavefaciens* and *Fibrobacter succinogenes*) and saccharolytic bacteria (*S. equinus* and *S. bovis*) in the hindgut of horses fed high-fibre or high-starch diets using faeces as a model for bacterial populations in the hindgut [12].

Materials and Methods

Feeding study and sample collection

Four mature horses (mares) of similar age (10 ± 2 years), size, breed (Welsh Cob) and BW (447 ± 80 kg) were used in a 4 × 4 Latin-square design consisting of four experimental periods of 31 days (28 days adaptation followed by 3 days of sampling). A wash out period of 5 days was included between experimental periods whereby ponies received a hay-only diet. The following diets were provided during the study: high-fibre with added Yea-Sacc (minimum guaranteed concentration 1 x 10^9 CFY/g: Alltech Inc., KY) live yeast (HFY); high-fibre without yeast (HF); high-starch with added yeast (HSY) and high-starch without yeast (HS). The high-fibre diets consisted of mature grass hay, fed at 1.75% body weight. High-starch diets consisted of a racing mix containing 340 g/kg DM (dry matter) of starch and fed in a 50:50 ratio with mature grass hay, to a total of 1.75% body weight. The chemical composition of the feedstuffs used in this study is provided inTable 1. Animals on the high-starch diet received 1.8 g starch per kg BW. The live yeast was added according to the recommended dosage of 4 g per day, fed once daily. The live yeast was added to the morning concentrate feed of the high-starch diet. For the high-fibre diet, the yeast was added to a small amount of chopped hay offered aa bucket feed. All diets were split into two meals per day (both hay and concentrate), fed at 8am and 4pm. Horses were individually housed in

loose boxes with water accessible *ad* libitum. Barn turnout was provided for at least 1 hour per day, to allow horses to exercise. Live-weight measurements were taken on a weekly basis for each individual to determine is any animals were in negative or positive energy balance. During the final 3 days of each experimental period, approximately 100 g of freshly voided faeces were collected at the same time daily prior to the 4 pm feed from each horse and stored separately at -20°C in labelled and sealed (air-tight) bags. At the end of each experimental period, faecal samples were pooled and a sub-sample (50 g) taken for analysis. All samples were stored at -20°C for later analysis.

Ethical approval was granted by the Royal (Dick) School of Veterinary Studies research ethics committee.

Total DNA extraction

DNA (total DNA) was extracted from the frozen faecal samples using the QIAamp® DNA stool kit (QIAGEN Ltd., UK), following the manufacturer's instructions, but with the addition of glass beads to aid the homogenisation of the samples [13]. Following DNA extraction and purification, the concentration of DNA in each sample was measured using a nanodrop and recorded before storage at -20°C until required.

Assessment of bacterial load

Samples were analyzed for the presence and abundance of specific fibrolytic and saccharolytic bacteria and total bacterial load. The bacteria tested for were: the fibrolytic bacteria *Ruminococcus flavefaciens* and *Fibrobacter succinogenes*; and the saccharolytic *Streptococcus equinus* and *Streptococcus bovis* (non-cellulolytic). PCR primers were designed using Primer Express® software (PE Applied Biosystems, UK) for the detection of

each of the target bacterial species, based on 16S rDNA sequences published in GenBank®. The Basic Local Alignment Search Tool (BLAST, National Centre for Biotechnology Information) was used to test the specificity of the probes. A previously published [14] universal primer set was utilised for total bacterial load quantification. Semi-quantitative real-time PCR was then performed on the extracted DNA, as described previously [15] using a Stratagene MX3000P Q-PCR system (Stratagene, UK). Primer used for the candidate bacteria are given in Table 2. The Ct values for each primer were measured and bacterial levels were determined relative to universal 16S by the delta Ct method [15].

Data handling and statistical analyses

Data were analysed in Minitab® using the General Linear Model (GLM) analysis of variance using the model: pony + period + (diet x treatment) . Least significant difference equations were used for the comparison between treatments. For all results, P values of < 0.05 were considered statistically significant.

Results

The DNA extraction method yielded relatively low (up to 39 μg/ml) concentrations of total DNA, with an average total DNA yield of 26 μg/ml. There was no difference in the total DNA extracted, despite the high-starch diets appearing to yield higher concentrations of DNA than the high-fibre (high fibre without yeast, HF, and high fibre diets, with mean values of 23 μg/ml and 24 μg/ml (for HF and HFY diets, respectively) compared to 30 μg/ml and 28 μg/ml (for HS and HSY diets respectively). Additionally, there was no difference in total DNA yield as a result of trial period or individual variation.

Trial period had no significant effect on levels of any target organisms. There was, however, a significant difference in levels of the target organisms associated with diet. *F.*

144 *succinogenes* was found to be the most abundant of the target species, with high relative
145 levels in all samples. Individual animal variation was very low for *F. succinogenes* (Figure
146 1).Diet did not appear to have any effect (P>0.05)) on the relative abundance of *R.*
147 *flavefaciens or F. succinogenes*. However, treatment with yeast led to a reduction (P<0.05) in
148 *F. succinogenes* in ponies fed the HS diet.

149 Conversely, diet was observed to have a considerable (P < 0.001) effect on relative
150 numbers of *S. bovis (P<0.001) and S. equinus (P<0.05)*. There was an increase in abundance
151 of these bacteria when horses were fed high starch diets compared to high fibre diets. There
152 was also significant variation (P = 0.049) in levels of *S. bovis* found in the faeces of
153 individual horses; two horses had lower relative levels of *S. bovis* when fed the HSY diet
154 compared to the HS diet. Individual variation between ponies in relative levels of this
155 bacterium was greater for the diets with added yeast than those without. *S. equinus* was
156 observed to have the lowest average abundance, although variation between individuals was
157 also high.

158

159 **Discussion**

160 F. succinogenes and R. Flavefaciens were selected as representative of fibrolytic bacteria
161 in horses, whilst S. bovis and S. equinus have been proposed as having a role in hindgut
162 acidosis and laminitis [1, 16, 17]. For the species targeted in this study, *F. succinogenes* was
163 found to be present at the highest relative levels in all individuals and during feeding of all
164 diets. Levels of this species appeared to be far greater than *R. flavefaciens*, previously
165 identified by Julliand et al. [17] as the most abundant species in the equine caecum. Lin and
166 Stahl [18] found substantial numbers of *F. succinogenes* in the equine colon, but far greater
167 numbers in the caecum, though they did not attempt to identify *R. flavefaciens* in their work,
168 as a comparison to *F. succinogenes*, despite its importance in fibre degradation in the horse.

In this study, the addition of yeast to the diet had no effect on levels of *R. flavefaciens* or F. *Succinogenes*. In a study by Grimm et al. [19] yeast supplementation was also found to have no effect on the microbial ecosystem of horses fed a high-fibre diet.

Feeding high starch diets resulted in increased numbers of *S. bovis* and a trend towards increased numbers of *S. equinus* compared to HF and HFY. This concurs with Medina et al. [20] who reported increased numbers of *Streptococci* with HS diets. The addition of yeast to the HS diet appeared to reduce relative amounts of *F. succinogenes* and *S. bovis* compared to the HS diet, which also concurs with reports of yeast supplementation limiting the extent of undesirable changes in the intestinal ecosystem of horses fed a high starch diet [20]

Individual variation was observed in relative levels of bacteria, particularly *S. bovis* and *S. equinus*. The high variation between individuals may have masked some possible effects of diet, particularly as there were only four horses used in this study. Individual variation in hindgut populations has been reported previously by Steelman et al. [21], who also used faecal sampling for their analysis of hindgut populations. Additionally, Mao et al. [22] described great variation in species present (again from faecal samples) in individual cattle during acidosis. Interestingly, the greatest individual variation observed here was in the two species known to be involved in lactic acidosis. It may be that those individuals harbouring larger relative numbers of these bacteria could be more susceptible to laminitis, although the disease was not induced in any horses in this study. This theory is supported by a recent *in vitro* study by Hale et al. [23] which used faecal inoccula from healthy horses and those with a history of laminitis. These authors found that gas production profiles during starch fermentation were much higher in horses with a history of laminitis than in normal horses, suggesting that the microbial community in horses which have had laminitis may be better

194 adapted to the breakdown of starch. It has been shown that the microbial community in
195 equine hindgut/faecal samples is highly diverse [24], which may example why some animals
196 are more responsive than other to yeast supplementation. The complete history of all the
197 horses in the present study is unknown and it is possible that one or more of these individuals
198 may have suffered from laminitis in the past.

199 The preservation method used (freezing) may have had an effect on the apparent
200 abundance of some species. For example, Hastie et al. [12] found that levels of
201 *R. flavefaciens* and *F. succinogenes* were higher when samples were lyophilized than when
202 they were simply frozen. These authors did not find the same trend in the case of *S. bovis*,
203 suggesting that sample preservation method can alter the apparent levels of some bacteria. It
204 could be that the gram-positive bacteria such as *S. bovis* are more resistant to damage when
205 frozen. These effects should be taken into account when assessing bacterial populations from
206 frozen faecal samples and may have had an impact on the results in this study. For example,
207 had lyophilisation been employed here, the relative levels of *R. flavefaciens* and *F.*
208 *succinogenes* might have been greater. Sample processing may also have affected bacteria
209 levels, temperature and storage time have been reported to impact on some bacterial counts
210 [25]. It is also possible that the DNA extraction method may be biased toward certain types
211 of bacteria, as some bacteria are more susceptible to chemical lysis. Indeed, a study by
212 Salonen et al. [26] demonstrated differences in yield of gram positive and gram negative
213 bacteria depending on extraction method, resulting in some bacterial species possibly being
214 represented to a larger or smaller proportional share than in reality. Glass beads were used in
215 addition to the chemicals provided in the extraction kit in an attempt to increase lysis
216 (physically) in cells that might not otherwise have been lysed effectively by the kit. It is
217 unknown what extent of bias may have been introduced by the method used in this study and
218 apparent levels of bacteria reported may not be a true reflection of the original community.

Other possible reasons for variable results obtained by molecular studies of bacterial communities in the equine hindgut could be the use of universal probe/primer sets which do not amplify all species present. In an ideal situation, a universal primer/probe set would be specifically designed for the detection of bacteria from the entire equine hindgut community. There is, however, still a lack of comprehensive knowledge of the entire microbial profile present and how this changes with diet.

Conclusion

The addition of live yeast (at the level used in this study) to a high-starch diet reduced the levels of *F. succinogenes*, but had no significant effect on the other candidate bacteria. High starch diets resulted in increased levels of *S. bovis* and a trend towards increased levels of *S. equinus* compared to high-fibre diets. Relative levels of *S. bovis* and *S. equinus* were observed to vary substantially between individual horses and the addition of yeast to the diet appeared to result in increased variability in numbers of *S. bovis*. The variation in levels of *Streptococcus spp.* between horses may have affected the results in this study, possibly masking any overall effects of diet. Future work could focus on assessing this variation and would likely involve a greater number of horses.

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References

[1] Rowe, J B, M J Lees, and D W Pethick. Prevention of acidosis and laminitis associated with grain feeding in horses. J. Nutr. 1994; 124: 2742S-2744S.

[2] Clarke, L L, M C Roberts, and R A Argenzio. Feeding and digestive problems in horses: Physiologic responses to a concentate meal. Vet Clin N AM-Equine 1990; 6(2): 433-451.

[3] Al Jassim, R A M and J B Rowe. A better understanding of acidosis and its control. Recent Advances in Animal Nutrition in Austrailia 1999; 12: 91-96.

[4] Pryde, S E, A J Richardson, C S Stewart, and H J Flint. Molecular analysis of the microbial diversity present in the colonic wall, colonic lumen and caecal lumen of a pig. Appl. Environ. Microbiol. 1999; 65: 5372-5377.

[5] Leser, T D, et al. Culture-independent analysis of gut bacteria: the pig gastrointestinal tract microbiota revisited. Applied and Environ. Microbiol. 2002; 68(2): 673-690.

[6] Tajima, K, et al. Rumen bacterial diversity as determined by sequence analysis of 16S rDNA libraries. FEMS Microbiol. Ecol. 1999; 29(2): 159-169.

[7] Tajima, K, et al. Diet-dependent shifts in the bacterial population of the rumen revealed with real-time PCR. Appl. Environ. Microbiol. 2001; 67(6): 2766-2774.

[8] Morgan, L M, J A Coverdale, M A Froetschel, and I Yoon. Effect of yeast culture supplementation on digestibility of varying forage quality in mature horses. J. Equine Vet. Sci. 2007; 27(6): 260-265.

[9] Agazzi, A, et al. Evaluation of the effects of live yeast supplementation on apparent digestibility of high-fiber diet in mature horses using the acid insoluble ash marker modified method. J. Equine Vet. Sci. 2001; 31(1): 13-18.

[10] Jouany, J P, B Medina, G Bertin, and V Julliand. Effect of live yeast culture supplementation on hindgut microbial communities and their polysaccharidase and

glycoside hydrolase activities in horses fed a high-fiber or high-starch diet. J. Anim. Sci. 2009; 87(9): 2844-2852.

[11] Milinovich, G J, et al. Fluorescence in situ hybridization analysis of hindgut bacteria associated with the development of equine laminitis. Environ. Microbiol. 2007; 9(8): 2090-2100.

[12] Hastie, P M, K Mitchell, and J M D Murray. Semi-quantitative analysis of Ruminococcus flavefaciens, Fibrobacter succinogenes and Streptococcus bovis in the equine large intestine using real-time PCR. Br. J. Nutr. 2008; 100: 561-568.

[13] Yu, Z and M Morrison. Improved extraction of PCR-quality community DNA from digesta and fecal samples. BioTech. 2004; 36: 808-812.

[14] Nadkarni, M A, E F Martin, M A Jacques, and N Hunter. Determination of bacterial load by real-time PCR using broad-range (Universal) probe and primer set. . Microbiology 2002; 148: 257-266.

[15] Benato, L, P M Hastie, P O'Shaughnessy, J M D Murray, and A Meredith. Effects of probiotic Enterococcus faecium and Saccharomyces cerevisiae on the faecal microflora of pet rabbits. J. Small Anim. Prac. 2014; 55(9): 442-446.

[16] Milinovich, G J, et al. Changes in equine hindgut bacterial popultation during oligofructose-induced laminitis. Environ. Microbiol. 2006; 8(5): 885-898.

[17] Julliand, V, A de Vaux, L Millet, and G Fonty. Identification of Ruminococcus flavefaciens as the Predominant Cellulolytic Bacterial Species of the Equine Cecum. Appl. Environ. Microbiol. 1999; 65(8): 3738-3741.

[18] Lin, C Z and D A Stahl. Taxon-specific probes for the cellulolytic genus Fibrobacter reveal abundant and novel equine-associated populations. Appl. Environ. Microbiol. 1995; 61(4): 1348-1351.

[19] Grimm, P, V Julliand, C Philippeau, and S Sadet-Bourgeteau. Effect of yeats supplementation on hindgut microbiota and digestibility of horses subjected to an abrupt chnage of hays. Livestock Sci. 2016; 186: 34-40.

[20] Medina, B, I D Girard, E Jacotot, and V Julliand. Effect of a preparation of *Saccharomyces cerevisiae* on microbial profiles and fermentation patterns in the large intestine of horses fed a high fibre or a high starch diet. J Anim Sci 2002; 80: 2600-2609.

[21] Steelman, S M, B P Chowdhary, S Dowd, J Suchodolski, and J E Janecka. Pyrosequencing of 16S rRNA genes in fecal samples reveals high diversity of hindgut microflora in horses and potential links to chronic laminitis. BMC Vet. Res. 2012; 8: 231.

[22] Mao, S Y, R Y Zhang, D S Wang, and W Y Zhu. The diversity of the fecal bacterial community and its relationship with the concentration of volatile fatty acids in the feces during subacute rumen acidosis in dairy cows. BMC Vet. Res. 2012; 8: 237.

[23] Hale, C E, H Warren, and A Hemmings. The fermentation of hay and starch when incubated in vitro with faecal inoccula from either normal healthy horses or horses with a history of laminitis. Forages and Grazing in Horse Nutrition 2012; 132: 357-361.

[24] Proudman, C J, et al. Characteristaion of the faecal metabolome and microbiome of thoroughbred racehorses. Equine Vet. J. 2015; 47: 580-586.

[25] Harlow, B E, T M Donley, L M Lawrence, and M D Flythe. Effect of starch source (corn, oats or wheat) and concentration on fermentation by equine faecal microbiota in vitro. J. Appl. Microbiol. 2015; 119: 1234-1244.

[26] Salonen, A, et al. Comparative analysis of fecal DNA extraction methods with phylogenetic microarray: Effective recovery of bacterial and archaeal DNA using mechanical cell lysis. J. Microbiol. Methods 2010; 81: 127-134.

Table 1 Chemical composition of the grass hay and high-starch cereal-mix (g/kg DM unless otherwise stated).

	Feedstuffs	
	Grass hay	**Cereal Mix**
Dry matter (g kg^{-1})	910	920
Organic matter	847	823
Crude protein	60	140
Water soluble carbohydrate	186	56
Starch	36	340
Acid detergent fibre	354	83
Neutral detergent fibre	613	196
Gross energy (MJ kg^{-1})	18.2	18.1

Table 2: Primers used for q-PCR

Bacteria	**Accession number**	**Forward Primer (5'-3')**	**Reverse Primer (5'-3')**
Universal	-	tcctacgggaggcagcagtgg	gccggtgcttcttctgcgg
R.flavefaciens	AF030447	gctggcggcacgcttaaca	gcggtacagttacattatgaggtattaccatcc
F.succinogenes	AJ496032	ccaacgcgcggttaatgtcc	ccaatgtggccgatcaccctc
S.bovis	AY442813	cgcgtaggtaacctgcctactagcg	ctagtgaagcaattgctcctttcaagca
S. equinus	JX123480	aagtggaacgcatgattgataccgg	caccgttcgcgactcatgattaa

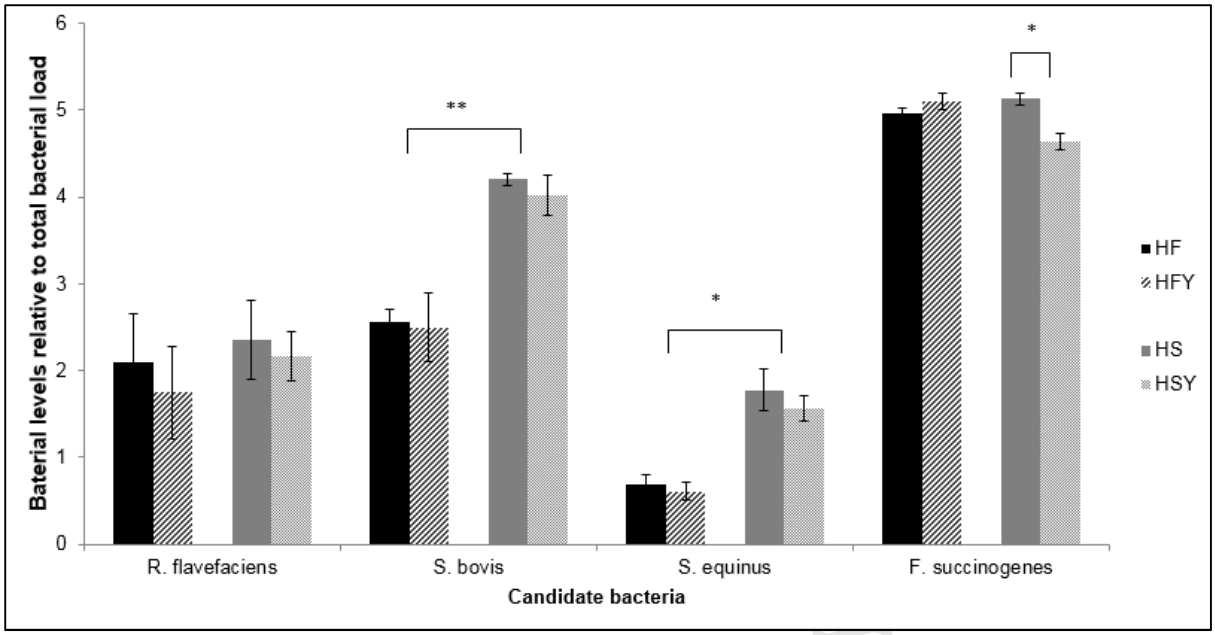

Figure 1: Semi-quantitative levels (± SE) of candidate bacteria in the faeces of horses fed a high-fibre (HF) or high-starch (HS) diet with (HYF and HSY) and without (HF and HS) yeast supplementation (n=4; * P<0.05, **P<0.01).

- We examined the effect of live yeast on equine large intestinal bacterial populations
- Horses were fed a low or high-starch diet
- Bacterial populations were affected by diet, but not by yeast supplementation