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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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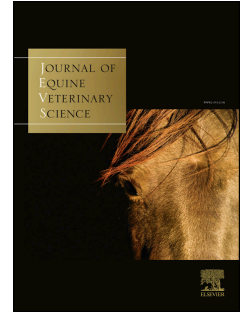
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# Accepted Manuscript

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

3

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14 Short title: Yeast Supplementation and Bacterial Populations in Equine Faeces

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25 **Abstract**

26 The objective of this study was to assess the effect of live yeast (*Saccharomyces cerevisiae*)  
27 supplementation on the populations of specific cellulolytic (*Fibrobacter succinogenes* and  
28 *Ruminococcus flavefaciens*) and saccharolytic (*Streptococcus equinus* and *Streptococcus*  
29 *bovis*) bacteria in the faeces of horses fed high-starch and high-fibre diets. Four horses were  
30 each fed diets consisting of high-fibre with no yeast (HF), high-fibre with yeast (HFY), high-  
31 starch with no yeast (HS) and high-starch with yeast (HSY) in a 4 × 4 Latin-square design  
32 study. Fresh faecal samples were collected on the last 3 days of each 31-day experimental  
33 period and were then assessed, using semi-quantitative real-time PCR, for total bacterial load  
34 and levels of target bacterial species, relative to the total bacterial load. The most abundant of  
35 the target species was *F. succinogenes* and the HSY diet resulted in a significant (P = 0.045)  
36 reduction in relative levels of this bacterium. No significant effect (P = 0.224) of diet was  
37 observed in relation to abundance of *R. flavefaciens*. Results show that diet did not have a  
38 significant (P = 0.068) effect on relative quantities of *S. equinus*, although there appeared to  
39 be a trend for increased levels of this bacterium during feeding of high starch diets. Numbers  
40 of *S. bovis* were higher (P < 0.001) when horses were fed HS and HSY diets than when fed  
41 HF and HFY diets. Significant variation in levels of *S. equinus* (P = 0.024) and *S. bovis* (P =  
42 0.049) was observed between individual horses.

43

## 44 **Introduction**

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

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

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

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

75

## 76 **Materials and Methods**

### 77 ***Feeding study and sample collection***

78 Four mature horses (mares) of similar age ( $10 \pm 2$  years), size, breed (Welsh Cob) and  
79 BW ( $447 \pm 80$  kg) were used in a  $4 \times 4$  Latin-square design consisting of four experimental  
80 periods of 31 days (28 days adaptation followed by 3 days of sampling). A wash out period of  
81 5 days was included between experimental periods whereby ponies received a hay-only diet.  
82 The following diets were provided during the study: high-fibre with added Yea-Sacc  
83 (minimum guaranteed concentration  $1 \times 10^9$  CFY/g: Alltech Inc., KY) live yeast (HFY);  
84 high-fibre without yeast (HF); high-starch with added yeast (HSY) and high-starch without  
85 yeast (HS). The high-fibre diets consisted of mature grass hay, fed at 1.75% body weight.  
86 High-starch diets consisted of a racing mix containing 340 g/kg DM (dry matter) of starch  
87 and fed in a 50:50 ratio with mature grass hay, to a total of 1.75% body weight. The  
88 chemical composition of the feedstuffs used in this study is provided in Table 1. Animals on  
89 the high-starch diet received 1.8 g starch per kg BW. The live yeast was added according to  
90 the recommended dosage of 4 g per day, fed once daily. The live yeast was added to the  
91 morning concentrate feed of the high-starch diet. For the high-fibre diet, the yeast was added  
92 to a small amount of chopped hay offered as a bucket feed. All diets were split into two meals  
93 per day (both hay and concentrate), fed at 8am and 4pm. Horses were individually housed in

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

102

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

105

#### 106 **Total DNA extraction**

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

112

#### 113 **Assessment of bacterial load**

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

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

127

### 128 ***Data handling and statistical analyses***

129 Data were analysed in Minitab<sup>®</sup> using the General Linear Model (GLM) analysis of  
130 variance using the model: pony + period + (diet x treatment) . Least significant difference  
131 equations were used for the comparison between treatments. For all results, P values of <  
132 0.05 were considered statistically significant.

133

### 134 **Results**

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

142 Trial period had no significant effect on levels of any target organisms. There was,  
143 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.

## 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 Jullian 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.

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

172 Feeding high starch diets resulted in increased numbers of *S. bovis* and a trend towards  
173 increased numbers of *S. equinus* compared to HF and HFY. This concurs with Medina et al.  
174 [20] who reported increased numbers of *Streptococci* with HS diets. The addition of yeast to  
175 the HS diet appeared to reduce relative amounts of *F. succinogenes* and *S. bovis* compared to  
176 the HS diet, which also concurs with reports of yeast supplementation limiting the extent of  
177 undesirable changes in the intestinal ecosystem of horses fed a high starch diet [20]

178

179

180 Individual variation was observed in relative levels of bacteria, particularly *S. bovis* and *S.*  
181 *equinus*. The high variation between individuals may have masked some possible effects of  
182 diet, particularly as there were only four horses used in this study. Individual variation in  
183 hindgut populations has been reported previously by Steelman et al. [21], who also used  
184 faecal sampling for their analysis of hindgut populations. Additionally, Mao et al. [22]  
185 described great variation in species present (again from faecal samples) in individual cattle  
186 during acidosis. Interestingly, the greatest individual variation observed here was in the two  
187 species known to be involved in lactic acidosis. It may be that those individuals harbouring  
188 larger relative numbers of these bacteria could be more susceptible to laminitis, although the  
189 disease was not induced in any horses in this study. This theory is supported by a recent *in*  
190 *vitro* study by Hale et al. [23] which used faecal inocula from healthy horses and those with  
191 a history of laminitis. These authors found that gas production profiles during starch  
192 fermentation were much higher in horses with a history of laminitis than in normal horses,  
193 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.

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

225

### 226 **Conclusion**

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

236

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240

241

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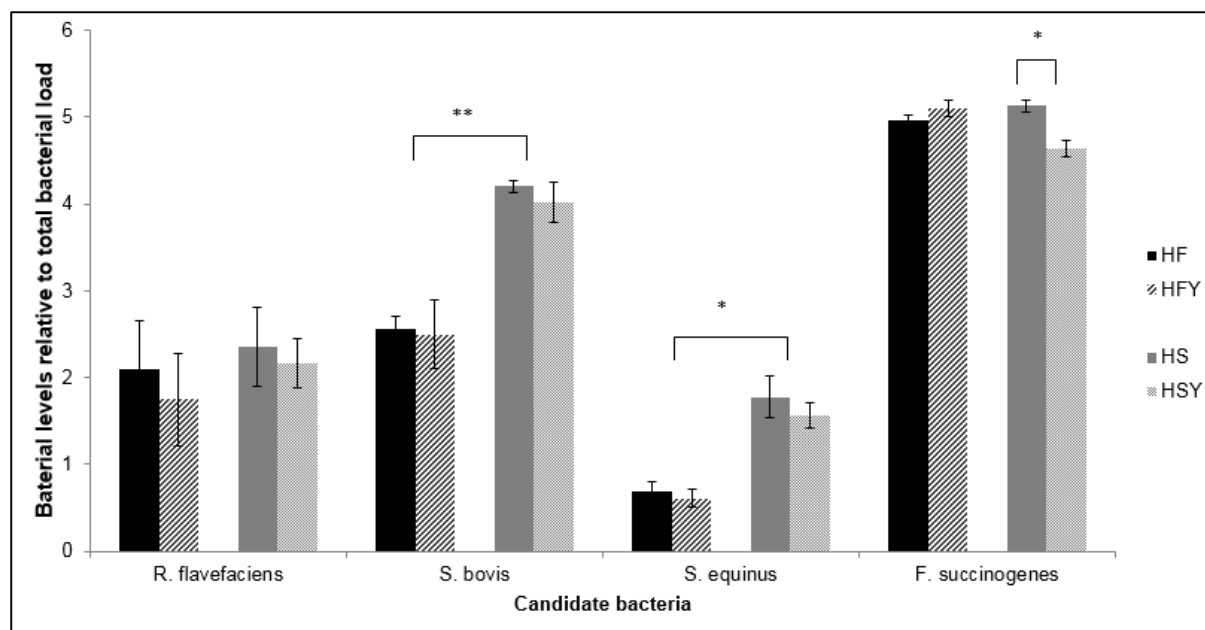


**Table 1** Chemical composition of the grass hay and high-starch cereal-mix (g/kg DM unless otherwise stated).

	Feedstuffs	
	Grass hay	Cereal Mix
Dry matter (g kg <sup>-1</sup> )	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 <sup>-1</sup> )	18.2	18.1

**Table 2:** Primers used for q-PCR

<b>Bacteria</b>	<b>Accession number</b>	<b>Forward Primer (5'-3')</b>	<b>Reverse Primer (5'-3')</b>
Universal	-	tctacgggaggcagcagtgg	gccggtgcttcttctgcgg
<i>R.flavefaciens</i>	AF030447	gctggcgccacgcttaaca	gcggtacagttacattatgaggtattaccatcc
<i>F.succinogenes</i>	AJ496032	ccaacgcgcggtaatgtcc	ccaatgtggccgatcacctc
<i>S.bovis</i>	AY442813	cgcgtaggtaacctgcctactagcg	ctagtgaagcaattgctccttcaagca
<i>S. equinus</i>	JX123480	aagtggaacgcatgattgataccgg	caccgttcgcgactcatgattaa



**Figure 1:** Semi-quantitative levels ( $\pm$  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

ACCEPTED MANUSCRIPT