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# The effect of supplementing pony diets with yeast on 1. *In vivo* and *in vitro* digestibility, faecal pH and particle size

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*Fibre is essential to maintain healthy gut; however, energy demands of performance horses can be too high to be met by forages alone. Yeast may support the function of cellulolytic bacteria to digest fibre. The aim of this work was to determine the effect of an oral supplement (VistaEQ) containing 4% live yeast on the* in vitro *and* in vivo *digestibility of high-starch (**HS**) and high-fibre diets (**HF**). Eight ponies were used in a 4 × 4 Latin square design consisting of 4- × 19-day periods and four diets: HF, HF + yeast (**HFY**), HS and HS + yeast (**HSY**).* In vivo *apparent digestibility (**AD**) was estimated using total collection technique, and faecal particle size was measured using NASCO digestive analyser. Faeces from the ponies were subsequently used as an inoculum in ANKOM RF gas production system to assess fermentation kinetics* in vitro*. Each module contained 1 g of feed substrate DM in the following combinations: 50% grass hay and 50% alfalfa **(HF_50 : 50)** or concentrate **(HS_50 : 50)**, and 75% grass hay and 25% alfalfa **(HF_75 : 25)** or concentrate **(HS_75 : 25)** with or without yeast. Yeast was able to induce more gas production from HF_75 : 25, HS_75 : 25 and HF_50 : 50 feed substrates incubated with respective faecal inoculum base. Yeast did not affect pH* in vitro *when the substrates were incubated in 50 : 50 ratio, while the pH was higher for HF_75 : 25 incubated with correspondent faecal inoculum compared to HS_75 : 25 and HSY_75 : 25. Yeast had no effects on ADF and CP AD of either diet. Yeast addition increased DM (HF: 0.2%, HS: 0.4%), organic matter (HF: 0.7%, HS: 1.3%), NDF (HF: 0.5%, HS: 1.5%), total detergent fibre (HF: 0.7%; HS: 0.4%) (*$P < 0.05$*) and also tended to increase hemicellulose AD (HF: 0.9%, HS: 1.2%) (*$P < 0.10$*). Faecal pH* in vivo *was higher for both HF diets compared to HS diet without yeast supplementation (*$P < 0.001$*, HF and HFY: 6.8; HS: 6.6, HSY: 6.7). However, no difference was observed in faecal pH when HSY was compared to both HF diets. Yeast had no effect on the size of the faecal particles (*$P > 0.05$*). Yeast increased* in vitro *gas production, suggesting more energy could be extracted from the feed, and the* in vivo *AD of some of the nutrients when HF and HS diets were fed.*

**Keywords:** *Saccharomyces cerevisiae*, gas production, high-fibre, high-starch, diet

## Implications

In the present study, yeast supplementation has improved the digestibility of the high-fibre and high-starch diets in ponies. The results suggest that improved fibre fraction digestibility with yeast supplementation may increase energy that ponies receive with the diet. Hence, VistaEQ yeast-containing supplement can be potentially used when lower nutritional quality forages are fed to equids. Moreover, it can be beneficial for horses and ponies with lower body condition score, hard keepers and performance horses with high energy requirements.

## Introduction

Horses are grazing and browsing hindgut fermenters adapted to eating plant-based diets continuously throughout the day. Fibre is essential to maintain healthy gut environment in horses; however, energy demands of performance horses can be too high to be met by forages alone, especially if the forage is of a poor quality (Harris *et al.*, 2016). Performance horse owners often restrict the amounts of forage fed to their horses, replacing it with high amounts of energy-dense feedstuffs such as cereal grains, despite the availability of better quality forages (Harris *et al.*, 2016). Thus, there is a need to develop alternative feeding strategies in order to meet nutrient and energy requirements of horses,

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while maintaining health and welfare as well as reduce negative effects of high-starch (**HS**) diets.

Digestive processes can be enhanced by a variety of approaches such as feedstuff selection and processing, improvements in forage quality, feeding management and the inclusion of digestion supplements such as probiotics and prebiotics. Many studies have reported the effects of yeast supplementation on *in vivo* and *in vitro* digestibility in horses, suggesting yeast may support the function of cellulolytic bacteria to digest fibre (Jouany *et al.*, 2008; Murray *et al.*, 2008; Jouany *et al.*, 2009; Agazzi *et al.*, 2011; Salem *et al.*, 2016; Murray *et al.*, 2017). The positive effects of yeast supplementation have been reported as improved fibre digestibility and increased feed efficiency (Agazzi *et al.*, 2011; Coverdale, 2016). Jouany *et al.* (2009) reported that most microbial enzymes involved in plant cell wall digestion increased after *Saccharomyces cerevisiae* was added to the diet, which may explain the increased fibre digestion in horses receiving yeast-supplemented diets. Conversely, some studies have reported no response to yeast supplementation, which could have been related to the dosage applied, the yeast strain used in the study, as well as the faecal sampling method employed (Taran *et al.*, 2016). In the rumen, yeast respiratory activity has been shown to protect anaerobic bacteria from oxygen, resulting in stimulated bacterial numbers *in vitro* (Newbold *et al.*, 2007). Mode of action for yeast supplements in equids is still relatively unknown and the evidence provided by previous research is inconsistent. The hindgut of the horse, which serves a fermentation chamber for fibre, is anatomically positioned after small intestine. Hence, less viable yeast cells may be able to reach the hindgut because of lower pH levels and enzymatic digestion encountered in the stomach and small intestine of the horse, compared to the rumen that is located prior the glandular stomach and small intestine in ruminants. Nevertheless, fibrolytic bacteria in the hindgut of the horse as well as rumen of the ruminants are known to be anaerobes (Shepherd *et al.*, 2012), thus yeast may support their function by oxygen scavenging effect in both animal species. The aim of the current experiment was to determine the effect of a yeast-containing feed supplement on *in vivo* digestibility, faecal pH and faecal particle size, when ponies were fed **HS** and high-fibre (**HF**) diets. A subsequent *in vitro* fermentation of the feedstuffs, DM loss (**DML**) and pH of the resultant suspension after incubation were also determined. The yeast supplement contained 4% live yeast *S. cerevisiae* ($5 \times 10^8$ colony-forming unit, CFU/g), 88% calcified seaweed and 8% limestone (VistaEQ, AB Vista, Marlborough, Wiltshire, UK).

## Material and methods

*Experiment 1 (*in vivo *total tract apparent digestibility, faecal pH and faecal particle size)*

*Experimental design.* Eight mature Welsh Section A pony geldings of 250 ± 12.0 kg (mean ± SEM) BW aged 5.3 ± 0.92 (mean ± SEM) years were used in a 4 × 4 complete randomised Latin square design consisting of 4- × 19-day periods. Each experimental period consisted of 14-day adaptation phase and 5-day recording phase followed by 7 days' wash out period.

*Diets and feeding.* The ponies were fed four different diets: HF, HF + yeast (**HFY**), HS and HS + yeast (**HSY**). The VistaEQ was mixed with alfalfa for the HFY diet and with the concentrate mix (**CM**) for the HSY diet. The diets were as follows:

HF: 75% grass hay (**GH**) and 25% chopped alfalfa (**CA**)

HFY: 75% GH and 25% CA supplemented with 50 g/pony per day of VistaEQ

HS: 75% GH and 25% CM

HSY: 75% GH and 25% CM supplemented with 50 g/pony per day of VistaEQ

The ratios were calculated on a **DM** basis to provide animals' a total daily DM intake of 17.5 g/kg BW. Table 1 shows the nutrient composition of the feedstuffs (alfalfa, CM and GH) and complete diets (HF, HFY, HS and HSY). Each pony

**Table 1** *Nutrient composition on DM basis of the feedstuffs and diets fed to ponies (*n = *7) during the* in vivo *experimental period in percentages (%) unless otherwise stated*

| | Feed | | | Diet | |
|---|---|---|---|---|---|
| Nutrient composition (%, unless otherwise stated) | CA | CM | Hay | HF/HFY | HS/HSY |
| DM | | | | | |
| Organic matter | 91.6 | 90.5 | 94.2 | 93.5 | 93.3 |
| Crude fibre | 26.8 | 11.5 | 36.1 | 33.8 | 29.9 |
| NDF | 41.2 | 37.1 | 76.6 | 67.8 | 66.7 |
| ADF | 34.6 | 22.2 | 44.5 | 42.0 | 38.9 |
| Hemicellulose | 6.6 | 14.9 | 32.1 | 25.8 | 27.8 |
| Total dietary fibre | 56.0 | 46.1 | 86.2 | 78.6 | 76.2 |
| CP | 17.3 | 10.9 | 8.4 | 10.6 | 9.0 |
| Acid ether extract | 1.9 | 3.1 | 1.5 | 1.6 | 1.9 |
| Starch | 1.7 | 25.4 | 0.0 | 0.4 | 6.4 |
| Gross energy (MJ/kg) | 18.1 | 17.3 | 18.4 | 18.3 | 18.1 |

CA = chopped alfalfa; CM = concentrate mix; HF = high fibre without yeast; HFY = high fibre with yeast; HS = high starch without yeast; HSY = high starch with yeast.

acted as its own control. Diets were fed in two meals per day 0900 and 1700 h.

*Management and exercise.* The animals were housed individually in stables (3.6 m × 5.6 m) with rubber matting and dust extracted pine wood shavings available during the adaptation phase and rubber matting only during the recording phase. Water was available *ad libitum* from automated drinkers. Ponies were exercised in an automated horse walker for 1 h each day during the adaptation period. During the collection phase, ponies were turned loose for 1 h per day in a concrete outdoor area.

*Measurements, sampling and data recording.* The total collection technique was performed to estimate the *in vivo* total tract apparent digestibility (**AD**) of the nutrients according to equation (1).

$$\text{Total tract apparent digestibility of nutrient (AD, \%)} = \frac{\text{nutrient intake} - \text{nutrient excretion}}{\text{nutrient intake}} \times 100 \quad (1)$$

Faecal and feed samples were analysed for DM, ash, CP, crude fibre, total dietary fibre, NDF, ADF, starch and gross energy. The feedstuffs were also analysed for acid ether extract (fat analysed by acid hydrolysis followed by ether extraction). Faecal pH was measured by obtaining faecal liquid after squeezing faeces through the double layer of muslin and measured using TRUEscience pH SMART Cap Kit with electrode and android tablet (TRUEscience, Over, Cambridge, UK).

*Faecal particle size analysis.* Particle size analysis of the faeces was carried out using a NASCO digestive analyser (Nasco, Fort Atkinson, WI, USA). The NASCO digestive analyser consists of three sieves: the top sieve with bigger holes (4.76 mm) retains faecal particles of a bigger size, the middle sieve with medium holes (2.38 mm) retains the particles of a medium size, while the bottom sieve with the smaller holes (1.59 mm) retains the fine particles. First, the complete NASCO digestive analyser and its individual parts were weighed. On day 5 of each collection period, a subsample of 500 g was added to the top sieve in small amounts to prevent blocking. After that, the sieve was washed through with a gentle flow of water until no more faecal particles were flowing through. Once the sample had completely separated, the weight of the complete digester and retained samples was measured to calculate the amount of faecal sample retained. Following that, the digester was dismantled and individual sieves together with retained samples were weighed. Using these data, the percentage of sample retained on each sieve was calculated and used for further statistical analysis.

*Statistical analyses.* Data were checked for normal distribution using the Anderson-Darling normality test. If data were not normally distributed, prior to the GLM procedure, the Box Cox transformation was performed. Data were analysed according to ANOVA GLM procedure (Minitab 17; Minitab Inc., State College, PA, USA). The model statement tested for the effects of 'diet', 'pony' and 'period' on AD with the pony as a random factor. The level of statistical significance was pre-set at <5%. Comparisons between treatment means were carried out with ANOVA GLM pairwise comparisons at 95% confidence intervals using the Tukey test.

*Experiment 2 (*in vitro *fermentation)*

*Substrates and experimental design.* Gas production modules contained one of eight feed substrate–faecal inoculum combinations as detailed in Table 2. Feed substrate–faecal inoculum combinations were as follows: HF inoculum (HFi)_50:50, HFYi_50:50, HSi_50:50, HSYi_50:50, HFi_75:25, HFYi_75:25, HSi_75:25 and HSYi_75:25. There were three replicate modules per feed substrate–faecal inoculum combination: 3 replicate modules × 8 substrate–inoculum combinations = 24 modules plus inoculum blanks (3 per inoculum type = 12 modules) gave a total of 36 modules.

Feedstuffs used as substrates for the *in vitro* study were the same as ponies were fed *in vivo*. The feedstuffs were ground to pass through a 1-mm screen prior to incubation. The ratios for each substrate type were calculated to give a total of 1 g of substrate DM. Faeces for faecal inoculum preparation (faeces inoculum base) were collected from each

**Table 2** In vitro *gas production study design employed to determine the effect of yeast supplementation (VistaEQ) to high-fibre and high-starch feed substrates incubated with an equine faecal inoculum*

| Substrate abbreviation | Substrate composition | Faecal inoculum base from ponies fed | Substrate + inoculum = treatment (abbreviation) |
|---|---|---|---|
| HF_50 : 50 | GH (50%) : GA (50%) | HF diet | HFi_50 : 50 |
| HF_75 : 25 | GH (75%) : GA (25%) | | HFi_75 : 25 |
| HFY_50 : 50 | GH (50%) : GA (50%) + VistaEQ (0.011 g) | HFY diet | HFYi_50 : 50 |
| HFY_75 : 25 | GH (75%) : GA (25%) + VistaEQ (0.011 g) | | HFYi_75 : 25 |
| HS_50 : 50 | GH (50%) : CM (50%) | HS diet | HSi_50 : 50 |
| HS_75 : 25 | GH (75%) : CM (25%) | | HSi_75 : 25 |
| HSY_50 : 50 | GH (50%) : CM (50%) + VistaEQ (0.011 g) | HSY diet | HSYi_50 : 50 |
| HSY_75 : 25 | GH (75%) : CM (25%) + VistaEQ (0.011 g) | | HSYi_75_25 |

HF = high fibre; HFY = high fibre yeast; HS = high starch; HSY = high starch yeast; GH = grass hay; GA = ground alfalfa; CM = concentrate mix.

pony after 2 weeks of adaptation to the diets (HF, HFY, HS and HSY). For each of the combination of feeds substrates incubated *in vitro*, the corresponding faecal inoculum base pooled from two ponies fed the same diet was used.

*Inoculum preparation and incubation.* The faeces were collected in 38°C preheated flasks, transported to the laboratory (<2 min) and stored at 38°C prior to inoculum preparation. The *in vitro* fermentation procedures were performed according to the modified protocol of Theodorou *et al.* (1994). Modification comprised reducing agent being added at 4 ml per 85 ml of culture media. Culture media (89 ml) was dispensed using an automated dispenser (MasterFlex Easy-Load II, Model 77200-62; Cole-Parmer, St Neots, Cambridgeshire, UK) into 250 ml coated ANKOM bottles (Systematic Instruments, Hazel Grove Stockport, UK). Faeces from each of the two ponies receiving the same diet were combined with an equal weight of anaerobic culture media. The same procedure was repeated four times, for each type of inoculum base. The faecal inoculum was dispensed immediately after preparation using 10 ml sterile syringes. Overall, each bottle contained 85 ml of media, 4 ml of reducing agent and 10 ml of inoculum. All bottles, except blanks, contained 1 g of substrate DM. The bottles were then purged with $CO_2$ for 15 s to replace the headspace volume. Bottles were closed with ANKOM modules tightly and placed in a water bath at 38°C prior to inoculation. Before the recording began, the valves were opened simultaneously to adjust the pressure inside the modules to ambient pressure. The bottles were incubated at 38°C until the end of the fermentation period.

*Gas measurements and analyses of vessel contents.* The automated ANKOM RF gas production system was used to assess the fermentation kinetics for 72 h, the pressure was automatically recorded every 10 min and gas accumulation measurements were performed according to ANKOM (2018) manual. Immediately after the fermentation period ended, contents of the bottles were analysed for DM content and the pH was measured using a TRUEscience SMART Bluetooth pH/Ion Meter Cap Kit with electrode and Android tablet (TRUEscience, Over, Cambridge, UK). The remaining culture fluid was separated from residual substrate material and adherent microbial biomass using a vacuum filtration system and pre-weighed sintered glass crucibles (porosity 1). The residue retained in each correspondent crucible was dried overnight in the oven at 60°C. Apparent DML was calculated as the difference between the DM remaining at the end of the fermentation period and initial DM of substrate incubated.

*Statistical analyses and modelling of gas accumulation profiles.* Parallel curve analysis was used to compare between substrates and treatments and it was performed according to Ross (1987). Gas production predicted according to exponential model (fitted equation) was as described below.

$$\text{Gas production} = A + b \times e^{-(\text{FRGP} \times \text{Time})} \quad (2)$$

where *FRGP* is fractional rate of gas production, *A* is predicted maximum gas production or the asymptote and *b* is curve shape parameter. The significance probability value associated with the *F* Value (F pr.) for regression was <0.001 and the $R^2 = 99.2$, indicating significant fit of the model. The rate constant was estimated according to the following equation:

$$\text{Curve shape parameter } (b) = Ae^{R \times L_T} \quad (3)$$

where *R* is the rate of degradation, *A* is predicted maximum gas production or the asymptote and $L_T$ is the time taken for gas production to start. Normal distribution for DML and pH *in vitro* were performed using Anderson-Darling test in Minitab 17 (Minitab 17; Minitab Inc.). One-way ANOVA "diet" *v.* "pH" or "DML" was performed followed by the Tukey *post hoc* test. The significance threshold was set at $P < 0.05$.

## Results

*Experiment 1 (*in vivo *digestibility, faecal pH and faecal particle size)*

One pony had a colic episode on day 5 of the first collection period fed HF diet and was excluded from the study. The rest of the ponies maintained good health throughout the study with the exception of one pony who was excluded from the trial before the fourth collection period due to the skin injury that required antibiotic treatment. Diets were well accepted by the animals. All the animals consumed the total amount of CM and alfalfa fed and hay refusals were negligible. Hence, the total DM intake was similar across all four diets.

*Total tract apparent digestibility.* The *in vivo* total tract AD of HF, HFY, HS and HSY diets are given in Table 3. Starch was not detected in the faeces (<0.1%); and hence, its digestibility was close to 100% on all diets. Yeast supplementation had no effect on ADF and CP AD of either diet; however, there was a general increase in AD for all the other nutrients with yeast supplementation.

Crude fibre digestibility of both HFY and HSY increased compared to HS diet alone and similar to HF diet ($P = 0.013$). Yeast supplementation increased AD of NDF when supplemented with HS diet ($P = 0.006$). Moreover, yeast supplementation tended to increase hemicellulose AD for both HS and HF diets ($P = 0.053$).

*Faecal pH* in vivo. Faecal pH *in vivo* when ponies were fed HS diet (pH = 6.6 ± 0.06), the pH was lower compared to the faecal pH when ponies were fed HF (pH = 6.8 ± 0.07) and HFY (pH = 6.8 ± 0.06) diets ($P = 0.018$) (Figure 1). However, the faecal pH when ponies were fed HSY diet (pH = 6.7 ± 0.04) was the same compared to both HF diets.

*Faecal particle size.* There were no differences in the percentage of faecal particles retained at top, medium or bottom

**Table 3** In vivo *total tract apparent digestibility (%) of the high-fibre (HF), high-fibre yeast (HFY), high-starch (HS) and high-starch yeast (HSY) diets in ponies* (n = 7)

| Apparent nutrient digestibility (%) | Diet | | | | *P* value |
|---|---|---|---|---|---|
| | HF | HFY | HS | HSY | |
| Dry matter | 49.2$^{c}$ | 49.4$^{bc}$ | 51.3$^{ab}$ | 51.7$^{a}$ | 0.003 |
| SEM | 0.47 | 0.47 | 0.69 | 0.67 | |
| Organic matter | 48.9$^{c}$ | 49.6$^{bc}$ | 51.6$^{ab}$ | 52.9$^{a}$ | <0.001 |
| SEM | 0.46 | 0.63 | 0.70 | 0.74 | |
| Crude fibre | 36.4$^{ab}$ | 36.3$^{a}$ | 33.3$^{b}$ | 38.1$^{a}$ | 0.013 |
| SEM | 1.35 | 1.39 | 1.55 | 1.78 | |
| NDF | 40.5$^{b}$ | 41.0$^{b}$ | 42.9$^{ab}$ | 44.4$^{a}$ | 0.006 |
| SEM | 0.47 | 0.84 | 0.70 | 1.20 | |
| ADF | 34.3 | 34.4 | 39.0 | 36.5 | 0.382 |
| SEM | 1.47 | 1.18 | 3.36 | 1.56 | |
| Hemicellulose | 50.8 | 51.7 | 54.3 | 55.5 | 0.053 |
| SEM | 2.16 | 2.13 | 1.55 | 2.71 | |
| Total dietary fibre | 46.6$^{b}$ | 47.3$^{ab}$ | 48.5$^{ab}$ | 48.9$^{a}$ | 0.031 |
| SEM | 0.69 | 0.81 | 0.98 | 0.91 | |
| CP | 58.0 | 60.6 | 57.2 | 58.4 | 0.325 |
| SEM | 1.43 | 0.91 | 0.86 | 1.38 | |
| Gross energy | 45.4$^{bc}$ | 44.7$^{c}$ | 47.6$^{ab}$ | 48.8$^{a}$ | <0.001 |
| SEM | 1.49 | 0.53 | 0.66 | 0.80 | |

$^{a,b,c}$Values within a row with different superscripts differ significantly at $P < 0.05$.

sieves of NASCO digestive analyser in response to the yeast supplementation, which is most probably due to the high variation within each dietary group (Table 4).

*Experiment 2 (*in vitro *fermentation)*

Simple exponential model was fitted and gas production curve fitted parameters were estimated and are presented in Table 5. Parallel curve analysis revealed that gas production was higher with yeast supplementation, irrespective of the substrate type and incubation ratio with only one exemption (Figure 2). When VistaEQ was added to HS feed substrate in the ratio of 50 : 50 (GH : CM), the gas production was higher from HSY feed substrate during the first half of the incubation period; however, the opposite trend was observed over the second part of the incubation period. The predicted maximum gas production or asymptotic gas production (ml/g DM incubated) was generally higher with yeast addition, except HSYi_50 : 50.

The effects of yeast supplementation on DML and pH of the culture medium following 72 h of *in vitro* incubation are shown in Table 6. End point digestibility measurements showed no increase in *in vitro* DML of treatments due to yeast addition. However, when treatments at a 50 : 50 ratio (50% GH and 50% CM or GA) were incubated, DML was lower for HFi_50 : 50 compared to both HFi_50 : 50 and HSYi_50 : 50 (P = 0.002). When treatments at a 75 : 25 ratio (75% GH and 25% CM or GA) were incubated, DML was lower for HFYi_75 : 25 compared to both HSi_75 : 25 and HSYi_75 : 25 (P = 0.008).

The pH of the culture media in the current experiment ranged from pH (mean ± SEM) 6.6 ± 0.01 to 6.9 ± 0.12. When treatments were incubated in a 50 : 50 ratio (50% GH and 50% GA or CM) pH *in vitro* for HFi_50 : 50 and HFYi_50 : 50 was higher than pH for HSi_50 : 50 and HSYi_50 : 50 ($P < 0.001$). When treatments were incubated in a 75 : 25 ratio (75% GH and 25% GA or CM), the pH of the resultant suspension was lower for HSi_75 : 25 and HSYi_75 : 25 compared to HFi_75 : 25 ($P = 0.014$); while the pH of HFYi_75 : 25 was not different from all the other treatments ($P > 0.05$).

## Discussion

*Experiment 1 (*in vivo *total tract apparent digestibility, faecal pH and faecal particle size)*

*Total tract apparent digestibility.* From the current results, yeast supplementation affected the degradation of both HS and HF diets. It would appear that yeast supplementation had greater effect on nutrient digestibility of HS diet: DM (HF: +0.2% *v.* HS: +0.4%), organic matter (HF: +0.7% *v.* HS: +1.3%), NDF (HF: +0.5% *v.* HS: +1.5%), total detergent fibre (HF: +0.7% *v.* HS: +0.4%) ($P < 0.05$) and hemicellulose AD (HF: +0.9% *v.* HS: +1.2%) ($P < 0.10$). It is generally postulated that yeast (the main component of VistaEQ) predominantly targets the fibrous fraction of the feedstuffs. However, yeast supplementation may mitigate the negative effects of HS diets on hindgut fibrolytic bacteria through competitive exclusion of less desirable bacteria, thus increasing the efficiency of fibrous fraction utilisation of HS diets (Callaway *et al.*, 2013). Competitive exclusion is a probiotic mechanism that involves, for example, yeast supplementation to animals with the aim to decrease the population of undesirable bacteria in the gastrointestinal tract (**GIT**). This theory can be supported by slightly increased faecal pH level when HS diet was supplemented with yeast (Figure 1).

The enhancement in the digestibility of the fibrous fraction of the diet with the addition of yeast supplements is consistent with the finding of the other researchers (Agazzi *et al.*, 2011; Salem *et al.*, 2016). Agazzi *et al.* (2011) report yeast supplementation improved AD of DM (+4.4%, $P = 0.03$), organic matter (+4.5% $P = 0.04$), NDF (+6.6%, $P = 0.04$) and ADF (+8.5%, $P = 0.03$). Agazzi *et al.* (2011) has reported greater increase in nutrient digestibility compared to the current study which may be attributed to greater amount of yeast supplemented per horse/day ($4.6 \times 10^{10}$ CFU/horse per day *v.* $1 \times 10^{9}$ CFU/pony per day), feeding yeast twice a day *v.* one time per day in the current study, use of horses *v.* ponies. Positive effects of yeast supplements on NDF AD (+6.7 to 15.5% depending on the supplement used) and ADF AD (+11.1 to 22.4%) of the horse have been also reported by Salem *et al.* (2016). Salem *et al.* (2016) have supplemented $2.25 \times 10^{11}$ CFU/horse per day, the dosage of which is 225 times greater than the one used in this study. In

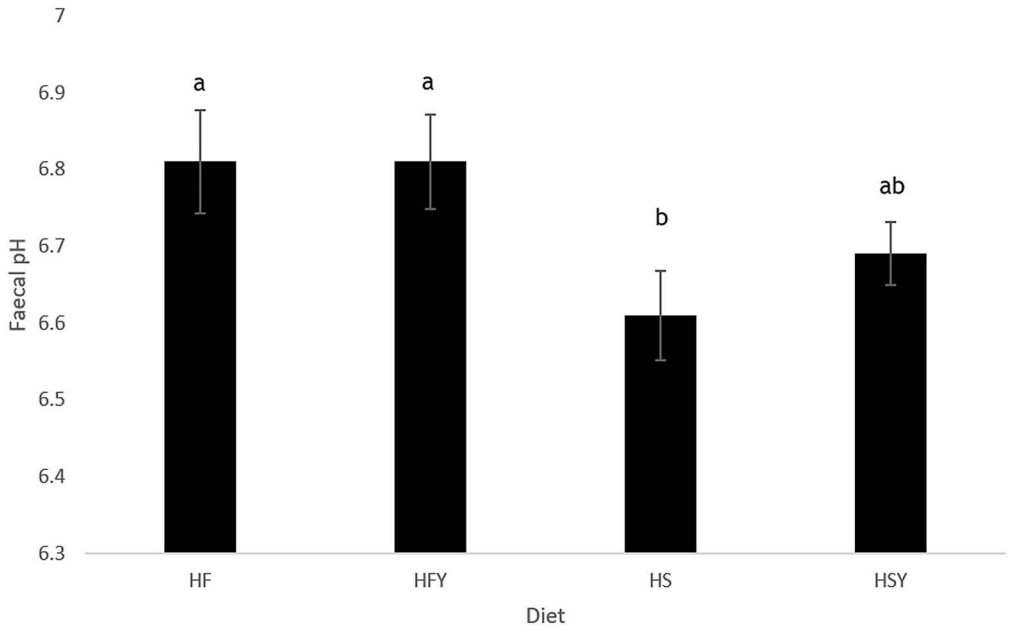


**Figure 1** Faecal pH *in vivo* when ponies ($n = 7$) were fed high-fibre (HF), high-fibre yeast (HFY), high-starch (HS) and high-starch yeast (HSY) diets. Error bars represent SEM.
Bars not sharing same superscript differ significantly.
HF: 75% grass hay (GH) and 25% chopped alfalfa (CA);
HFY: 75% GH and 25% CA supplemented with 50 g of VistaEQ (yeast-containing supplement)
HS: 75% GH and 25% concentrate mix (CM);
HSY: 75% GH and 25% CM supplemented with 50 g of VistaEQ

**Table 4** *Distribution of the faecal particles > 1.59 mm retained at each sieve of NASCO digestive analyser when ponies (n = 7) where fed high-fibre (HF), high-fibre yeast (HFY), high-starch (HS) and high-starch yeast (HSY) diets*

| | Diet | | | | |
|---|---|---|---|---|---|
| Particle size (mesh size (%)) | HF | HFY | HS | HSY | *P* value |
| Coarse (4.76 mm) | | | | | |
| Mean | 72.0 | 64.8 | 66.6 | 59.9 | 0.640 |
| SEM | 4.60 | 7.71 | 8.68 | 8.78 | |
| Medium (2.38 mm) | | | | | |
| Mean | 13.1 | 15.4 | 15.6 | 16.9 | 0.579 |
| SEM | 3.72 | 5.46 | 5.23 | 4.80 | |
| Fine (1.59 mm) | | | | | |
| Mean | 14.8 | 19.6 | 17.6 | 23.1 | 0.548 |
| SEM | 2.62 | 4.14 | 3.98 | 4.60 | |

HF: 75% grass hay (GH) and 25% chopped alfalfa (CA).
HFY: 75% GH and 25% CA supplemented with 50 g of VistaEQ (yeast-containing supplement).
HS: 75% GH and 25% concentrate mix (CM).
HSY: 75% GH and 25% CM supplemented with 50 g of VistaEQ.

the current study, statistically significant effect has been seen at lower levels of supplementation. Further research is required to test higher levels of supplementation, use of horses instead of ponies and feeding yeast twice daily. Nevertheless, some studies showed no effect of yeast on nutrient AD whatsoever (Mackenthun *et al.*, 2013; Taran *et al.*, 2016). Taran *et al.* (2016) supplemented $5 \times 10^9$ to $1.5 \times 10^{10}$ CFU/miniature horse per day whose dosages were higher than those used in the current experiment and reports reduced CP digestibility with yeast supplementation and no effect of yeast on digestibility of other nutrients. Mackenthun *et al.* (2013) supplemented $2 - 6 \times 10^{10}$ CFU/horse per day and report no changes associate with yeast addition to the diet. Differences in the results of various experiments involving yeast supplementation may be attributed to the use of different strain of *S. cerevisiae*, different horse breeds, dosage, amount of viable cells, yeast administration technique and frequency, diet composition, number of meals per day, part of the digestive tract from where digesta was obtained for the analysis and the analyses performed.

Yeast can reach and survive in the caecum and the right ventral colon of the horse (Medina *et al.*, 2002; Jouany *et al.*, 2008; Jouany *et al.*, 2009) but have a limited ability to colonise them and thus has to be supplemented regularly with the diet (Schoster *et al.*, 2014). Enhancement of dietary nutrient digestibility through yeast supplementation can be attributed to the fact that a major amount of ingested yeast can survive during transition through the GIT of the horse and reach the caecum and colon, resulting in an increase in the digestibility of the fibre fractions (Medina *et al.*, 2002). This may be accredited to the stimulation of microbial cellulolytic activity in the hindgut (Jouany *et al.*, 2009).

*Faecal pH.* pH is a commonly used parameter in equine nutrition studies to predict the GIT health after feeding different diets (Hydock *et al.*, 2014). However, faecal pH does not appear to indicate short-term variations in the hindgut and

**Table 5** *The effect of yeast (VistaEQ) supplementation of the high-fibre (HF) and high-starch (HS) substrates on gas production curve fitted parameters: predicted maximum gas production (A), fractional rate of gas production (FRGP), time taken to produce 50% of total gas production ($T_{50}$) and Lag time ($L_T$) for eight substrate combinations when incubated with equine faecal inoculum*

| | Treatment | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Parameters | HFi_50 : 50 | HFYi_50 : 50 | HFi_75 : 25 | HFYi_75 : 25 | HSi_50 : 50 | HSYi_50 : 50 | HSi_75 : 25 | HSYi_75 : 25 |
| *A* (ml/g DM) | | | | | | | | |
| Mean | 122 | 131 | 137 | 144 | 148 | 144 | 141 | 146 |
| SEM | 0.2 | 0.1 | 0.3 | 0.1 | 0.1 | 0.1 | 0.2 | 0.2 |
| FRGP (1/h) | | | | | | | | |
| Mean | 0.064 | 0.088 | 0.057 | 0.081 | 0.080 | 0.095 | 0.070 | 0.079 |
| SEM | 0.0006 | 0.0003 | 0.0005 | 0.0005 | 0.0003 | 0.0004 | 0.0005 | 0.0007 |
| $T_{50}$ (h) | | | | | | | | |
| Mean | 10.8 | 7.8 | 12.1 | 8.6 | 8.6 | 7.3 | 9.9 | 8.8 |
| SEM | 0.11 | 0.03 | 0.11 | 0.05 | 0.03 | 0.03 | 0.07 | 0.08 |
| $L_T$ (h) | | | | | | | | |
| Mean | 0.704 | −0.855 | 1.422 | −0.354 | −0.591 | −0.703 | 0.139 | −0.282 |
| SEM | 0.0819 | 0.024 | 0.0821 | 0.039 | 0.0204 | 0.022 | 0.0551 | 0.0599 |

HFi_50 : 50: 50% grass hay (GH) and 50% ground alfalfa (GA) + inoculum base from ponies fed high-fibre (HF) diet (HFi).
HFYi_50 : 50: 50% GH and 50% GA supplemented with 0.011 g of VistaEQ + inoculum base from ponies fed high-fibre yeast (HFY) diet (HFYi).
HSi_50 : 50: 50% GH and 50% concentrate mix (CM) + inoculum base from ponies fed high-starch (HS) diet (HSi).
HSYi_50 : 50: 50% GH and 50% CM supplemented with 0.011 g VistaEQ + inoculum base from ponies fed high-starch yeast (HSY) diet (HSYi).
HFi_75 : 25: 75% GH and 25% GA + HFi; HFYi_75 : 25: 75% GH and 25% GA supplemented with 0.011 g of VistaEQ + HFYi.
HSi_75 : 25: 75% GH and 25% CM + HSi; HSYi_75 : 25: 75% GH and 25% CM supplemented with 0.011 g of VistaEQ + HSYi.

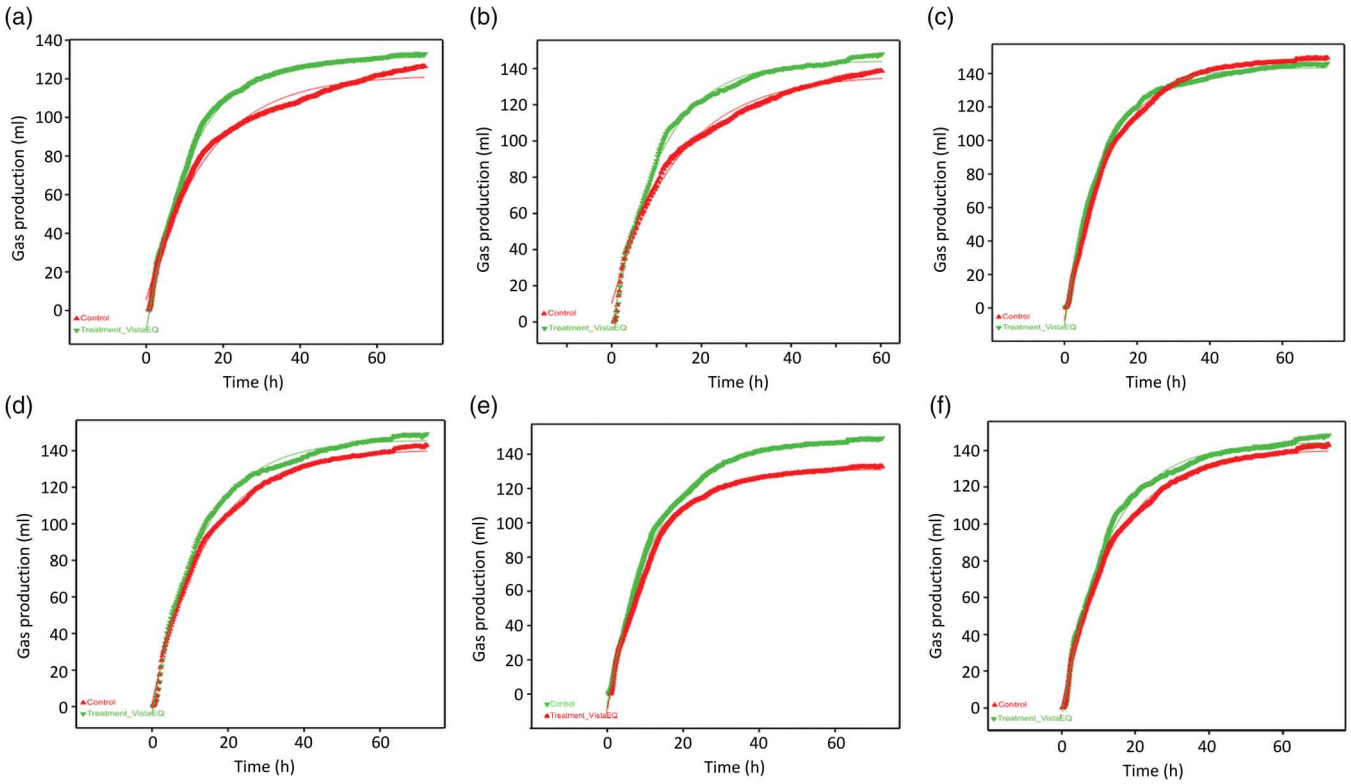


**Figure 2** Fitted and observed relationship between gas production (ml) from the substrates incubated with an equine faecal inoculum with and without yeast (VistaEQ) supplementation. (a) HFi_50 : 50 (control: 50% hay, 50% alfalfa) and HFYi_50 : 50 (treatment: 50% hay, 50% alfalfa + 0.011 g VistaEQ). (b) HFi_75 : 25 (control: 75% hay, 25% alfalfa) and HFYi_75 : 25 (treatment: 75% hay, 25% alfalfa + 0.011 g VistaEQ). (c) HSi_50 : 50 (control: 50% hay and 50% concentrate mix) and HSYi_50 : 50 (treatment: 50% hay and 50% concentrate mix + 0.011 g VistaEQ). (d) HSi_75 : 25 (control: 75% hay and 25% concentrate mix) and HSYi_75 : 25 (treatment: 75% hay, 25% concentrate mix + 0.011 g VistaEQ). (e) HSi_50 : 50 (control: 50% hay and 50% concentrate mix) and HFYi_50 : 50 (treatment: 50% hay and 50% alfalfa + 0.011 g VistaEQ). (f) HSi_75 : 25 (control: 75% hay and 25% concentrate mix) and HFYi_75 : 25 (treatment: 75% hay and 25% alfalfa + 0.011 g VistaEQ) over time (h).

**Table 6** *Dry matter loss (DML) and pH for high-fibre (HF) and high-starch (HS) substrates incubated with equine faecal inoculum at a 50 : 50 and 75 : 25 ratios* in vitro *with or without yeast (VistaEQ) addition*

| | Treatment | | | | |
|---|---|---|---|---|---|
| Ratio | HFi_ | HFYi_ | HSi_ | HSYi_ | *P* value |
| 50 : 50 | | | | | |
| DML (%) | | | | | |
| Mean | 41.6[a] | 43.0[ab] | 55.6[bc] | 57.1[c] | 0.002 |
| SEM | 2.00 | 1.32 | 0.30 | 0.19 | |
| pH | | | | | |
| Mean | 6.89[a] | 6.68[a] | 6.59[b] | 6.56[b] | <0.001 |
| SEM | 0.119 | 0.010 | 0.022 | 0.011 | |
| 75 : 25 | | | | | |
| DML (%) | | | | | |
| Mean | 43.6[ab] | 42.5[a] | 51.1[bc] | 51.5[c] | 0.008 |
| SEM | 0.78 | 3.52 | 0.21 | 0.56 | |
| pH | | | | | |
| Mean | 6.64[a] | 6.61[ab] | 6.58[b] | 6.58[b] | 0.014 |
| SEM | 0.015 | 0.010 | 0.014 | 0.017 | |

[a,b,c]Values within a row with different superscripts differ significantly at $P < 0.05$.
HFi_50 : 50: 50% grass hay (GH) and 50% ground alfalfa (GA) + inoculum base from ponies fed high-fibre (HF) diet (HFi).
HFYi_50 : 50: 50% GH and 50% GA supplemented with 0.011 g of VistaEQ + inoculum base from ponies fed high-fibre yeast (HFY) diet (HFYi).
HSi_50 : 50: 50% GH and 50% concentrate mix (CM) + inoculum base from ponies fed high-starch (HS) diet (HSi).
HSYi_50 : 50: 50% GH and 50% CM supplemented with 0.011 g VistaEQ + inoculum base from ponies fed high-starch yeast (HSY) diet (HSYi).
HFi_75 : 25: 75% GH and 25% GA + HFi.
HFYi_75 : 25: 75% GH and 25% GA supplemented with 0.011 g of VistaEQ + HFYi.
HSi_75 : 25: 75% GH and 25% CM + HSi.
HSYi_75 : 25: 75% GH and 25% CM supplemented with 0.011 g of VistaEQ + HSYi.

as such it cannot be used as an indicator of variations in caecal pH induced by a single meal. In the current study, faecal pH was measured after a relatively long adaptation period and, therefore, is more likely accurately to reflect the overall effect of the diet on the hindgut environment. The current experiment shows a positive change in pH with yeast supplementation of the HS diet. Even though this change was statistically significant, it should be noted that the faecal pH of ponies on all four diets remained within the typical physiological range with very little numerical variation (pH 6.61 to 6.81).

*Faecal particle size.* In the current study, there were no differences in the percentage of distribution of larger faecal particles with yeast supplementation. One possible reason may be that in wet sieving the amount of water retained with each fraction cannot be avoided. The average for faecal particle size (all particles down to 0.16 mm) in the horse is reported to be 1.8 ± 0.8 mm; however, the proportions of faecal particles retained on each sieve vary a lot depending on the diet and the methods employed/apparatus used (Grenet *et al.*, 1984). In horses, faecal particle size has been recently evaluated in relation to dental pathologies and colon impactions (Zwirglmaier *et al.*, 2013; Gunnarsdottir *et al.*, 2014), DM digestibility (**DMD**) *in vivo* (Martuzzi *et al.*, 2015) as well as dietary particle size (Lapinskas *et al.*, 2017). Zwirglmaier *et al.* (2013) determined feed and faecal particle size for horses with mild to moderate dental findings and found no signs of discomfort when chewing before and after dental correction. The authors report no effect of dental correction of faecal particle size and improved digestibility of some of the nutrients. Similarly, Gunnarsdottir *et al.* (2014) reported no association between dental pathology and faecal particle size. However, in horses with large colon impaction, faecal particle size was positively correlated with periodontal disease index. The overall faecal particle size for the control group (1.7 ± 0.4 mm) was significantly higher than for the colic group (1.3 ± 0.4 mm). The study of Wickström (2010) suggests that higher DMD is associated with greater amounts of smaller faecal particles and *vice versa*. Faecal particle size analysis can provide valuable information on feedstuff degradation in the GIT of the horse when employed together with total collection technique and other methods enabling the assessment of nutritive value of diets for horses as well as microbiota assessment. Determination of faecal particle size using NASCO digestive analyser provides variable results and may reflect other factors unrelated to gut microbial activity such as diet particle size, health state and individual variability.

*Experiment 2 (*in vitro *fermentation)*

*Gas production parameters.* The yeast inclusion rate in the current study was 0.011 g/1 g feed substrate DM, which is similar to the commercial recommended daily inclusion rates for use *in vivo*. Generally, VistaEQ supplementation increased gas production from the HF and HS feed substrates (Figure 2).

However, gas production was higher from HSYi_50 : 50 compared to HSi_50 : 50 during the first part of the incubation period; while the opposite trend was observed over the second part of the incubation period. Reduced gas production from HSYi_50 : 50 compared to HSi_50 : 50 during the second part of the incubation may be due to a reducing amount of fibre in HSYi_50 : 50 for yeast to act upon. Yeast is known to target the fibrous fraction of feeds (Monroy Salazar *et al.*, 2016), thus a similar effect of increased gas production as with 75 : 25 (GH : CM) ratio was not observed in the second part of the incubation process. Nevertheless, the first part of incubation has more physiological importance. The measurements that exceed average retention time in the GIT of the horse, which vary from 31.7 h (HF diet) to 35.0 h (HSY diet) according to Jouany *et al.* (2008), have little biological relevance but are necessary for model fitting. Yeast supplementation increased *in vitro* gas production of HFYi_75 : 25 to levels higher than those produced by the HSi_75 : 25 but not gas production of HFYi_50 : 50 over those produced by HSi_50 : 50. Again, this finding may be attributed to insufficient fibre content in 50 : 50 ratio, possibly showing that yeast did not have enough fibrous substrate to act upon it as alfalfa had a lower content of crude fibre, NDF, ADF and total digestible fibre compared to the GH. From a physiological perspective, the 75 : 25 ratio is much closer to the commonly employed equine feeding practices compared to the 50 : 50 ratio; it is more representative of practice and thus of scientific interest. As discussed above, yeast may be useful when feeding HS diets due to a competitive exclusion mechanism (Callaway *et al.*, 2013); however, feeding less than 15 g of forage DM/kg horse BW is not recommended (Harris *et al.*, 2016); hence, a ratio of 50% forage and 50% concentrate is generally not advisable for horses.

In the current study, the inoculum base was obtained from the ponies fed diet representing feed substrate combinations used for *in vitro* incubation. Thus, bacteria present in the inoculum base were adapted to the feed substrate encountered in the *in vitro* fermentation process, which is not always implemented in the experimental design of *in vitro* gas production studies (Murray *et al.*, 2005; Elghandour *et al.*, 2014). The volume of gas produced during *in vitro* fermentation reflects the fermentation activity of the inoculum used in each case and the potential of *S. cerevisiae* to further enhance such gas production (Elghandour *et al.*, 2016). An inoculum origin is important to consider when designing *in vitro* studies.

*Dry matter loss and pH.* The pH of the culture media in the current experiment ranged from 6.56 to 6.89 at the end of the incubation period, remaining within the physiological levels typically encountered in the hindgut of the horse as the fibre fermentation in the hindgut of the horse is efficient at pH 6 to 7 (Sjaastad *et al.*, 2010). Richards *et al.* (2006) report that pH lower than 6.2 is considered below optimal level for cellulolytic and lactate utilising bacteria to proliferate, suggesting a pH below 6.2 may reduce fibre digestibility, while Williamson *et al.* (2007) suggests that faecal pH below 6.32 being acidic. However, the pH values obtained in the *in vitro* gas production system experiments may not entirely reflect the *in vivo* situation since the culture medium used in the gas production method of Theodorou is heavily buffered (Theodorou *et al.*, 1994). Conversely, the small intestine neutralises the pH of the ingesta coming from stomach via pancreatic bicarbonate and hepatic bile salts (Ericsson *et al.*, 2016), thus also having a heavy buffering effect on gut digesta. Faecal pH *in vivo* ranged from 6.61 (HS diet) to 6.81 (HFY diet), while the pH *in vitro* ranged from 6.56 (HSYi_50 : 50) to 6.89 (HFi_50 : 50 ratio). Overall, the pH values *in vitro* were similar to the pH values determined *in vivo*. The pH *in vitro* did not reflect differences between substrates in a similar way to that observed in the *in vivo* results.

Differences between pH levels *in vivo* and *in vitro* may also be due to the intestinal absorption of fermentative end products, which takes place in live animal but not *in vitro* (Garber *et al.*, 2017). Hence, there is a need to assess the *in vitro* results alongside the *in vivo* results. Figure 3 compares DMD and pH *in vivo and in vitro.*

In regions where the quality of GH fed to horses is generally low due to climatic conditions, there is a need to develop alternative feeding techniques that would increase the nutritive value of diets without compromising digestive health. Another key issue in equine nutrition science is to reduce the negative effects of starch-rich cereal grains, which may cause microbial and, hence, digestive upsets in the horse. Probiotics, such as yeast, are generally easy to use for the horse owners, can be administrated in small dosages mixed with other feed meals, are long-lasting and are relatively inexpensive.

There is a limited amount of research comprehensively studying the effects of yeast supplementation on *in vivo* total tract nutrient digestibility, faecal particle size and pH, fermentative parameters *in vitro*, and on faecal microbiota investigated within the same experimental framework. The information previously reported suggests that yeast supplementation was not always able to induce positive changes for equine gastrointestinal health. Thus, every new strain of yeast prior to claiming a certain effect needs to be researched.

In the current study, DMD *in vivo* and DML *in vitro* followed the same trend: DMD *in vivo* was generally higher for both HS diets compared to both HF diets, which concurred with *in vitro* results. However, Lowman *et al.* (1999) reported that agglutination of feedstuffs during incubation may occur and cause poor filtration quality and lead to erroneous results, suggesting that determination of DML as a single predictor gives poor estimates of *in vivo* digestibility. Hence, *in vitro* methods measure maximum digestibility and can be closer to true digestibility (Blummel *et al.*, 1997). The addition of *S. cerevisiae* into the system could have resulted in increased cellulolytic bacteria microbial mass since DML in the *in vitro* gas production experiments is apparent DML due to the microbial biomass adhering to the residue (Murray *et al.*, 2008). According to Schoster *et al.* (2014), live yeast mechanisms of action may be attributed to immune

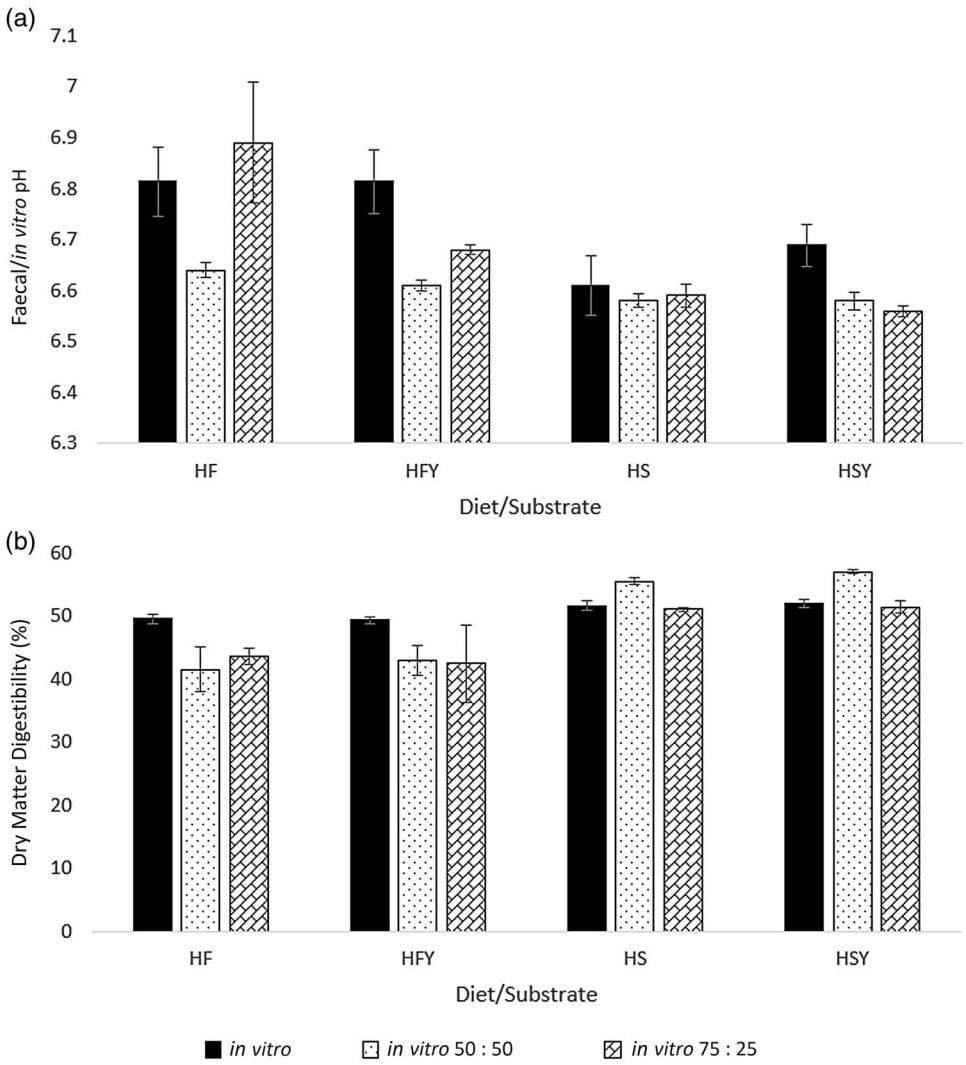


**Figure 3** A, Faecal pH *in vivo* when ponies ($n = 7$) were fed high-fibre (HF), high-fibre yeast (HFY), high-starch (HS) and high-starch yeast (HSY) diets and the pH of the resultant suspension after *in vitro* incubation of treatments (HF, HFY, HS and HSY feed substrates incubated with correspondent faecal inoculum in 50 : 50 and 75 : 25 ratios) for 72 h. B, Dry matter digestibility (DMD) *in vivo* when ponies ($n = 7$) were fed high-fibre (HF), high-fibre yeast (HFY), high-starch (HS) and high-starch yeast (HSY) diets and DMD after *in vitro* incubation of treatments (HF, HFY, HS and HSY feed substrates incubated with correspondent faecal inoculum in 50 : 50 and 75 : 25 ratios) for 72 h.
Error bars represent SEM.
HF: 75% grass hay (GH) and 25% chopped alfalfa (CA).
HFY: 75% GH and 25% CA supplemented with 50 g of yeast-containing supplement (VistaEQ) *in vivo* or 0.011 g *in vitro*.
HS: 75% GH and 25% concentrate mix (CM).
HSY: 75% GH and 25% CM supplemented with 50 g VistaEQ *in vivo* or 0.011 g *in vitro*.

modulation, antimicrobial production, competitive exclusion and inactivation of bacterial toxins. Parts of the yeast such as yeast cell walls may be utilised by host microorganisms, serving a source of nutrients and vitamins for beneficial gut bacteria and thus influencing gastrointestinal microbiota (Garber *et al*., 2020).

Gas production and fermentation kinetics of HF and HS feed substrates were generally increased with yeast supplementation. This finding is supported by *in vivo* nutrient digestibility results showing that some of the fibre fractions' total tract AD increased with yeast supplementation. Faecal pH increased when the ponies were fed a higher starch diet; however, the faecal pH *in vivo* and pH of the resultant suspension *in vitro* did not follow the same trend. The addition of yeast to the diet did not show any effect on the observed faecal particle size.

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### Declaration of interest

The authors have declared that no competing interests exist

### Ethics statement

The institutional and national guidelines for the care and use of animals were followed and all experimental procedures involving animals were approved by the University of Glasgow, School of Veterinary Medicine research ethics committee, reference: 05a/14.

### Software and data repository resources

None of the data were deposited in an official repository.

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