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Straight from the horse's mouth: The effect of different feedstuffs on oral pH in horses and ponies

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ABSTRACT

Feedstuffs, especially ensiled forage, may be detrimental to equid oral health by exposing the oral cavity to low pH. This study aimed to identify if salivary pH was altered by 1) a range of different feedstuffs and (2) specifically by feeding haylages with differing nutrient profiles. Two studies were conducted. The first measured oral pH following five feedstuffs, (hay, haylage, unmolassed sugarbeet pulp, unmolassed alfalfa chaff and rolled oats), tested individually over five days. Saliva (≥1 ml) was collected in triplicate, prior to feeding, directly after ingesting 500 g of each feedstuff, then 15 min and 30 min post-prandially. Oral pH was determined (pH meter) within 10 min of collection. In study two, eight ponies, were fed as their total diet, four different haylages over four 15-day periods. Saliva was collected, prior to feeding and immediately after ingesting 500 g of forage on day 1, day 6, and day 12 of each period. Samples were collected and analysed as per study one. All data were analysed by repeated measures ANOVA, and in study two linear regression was used to attempt to predict nutrients that influenced oral pH. All statistics were conducted in Genstat 20 th Ed. Only feeding unmolassed sugarbeet caused a reduction ($p<0.001$) in oral pH. There were differences in oral pH depending on the type of haylage fed in study two but at all times oral pH post-feeding was the same or greater than basal pH. These studies suggest any feed associated modulation of oral pH in horses may only be short-lived and quickly buffered by saliva. However, these studies only reflect oral pH within the oral cavity around the feeding occasion and may not reflect gingival pH or the effects of different feeds over longer time periods.

1. Introduction

There is little data on the effect of feeding on the pH of the equid oral cavity. The primary contributors to saliva in equids are the parotid, submandibular and sublingual salivary glands, producing ~35-40 litres of saliva per day [1]. Equid saliva is >99 % water containing electrolytes, bicarbonate and previously reported as typically having a pH 7.49-9.1 [1]. Saliva is only produced upon chewing in horses [1] playing an important role in the formation of a moist bolus during the ingestion of feedstuffs. In addition, by buffering the oral pH, saliva helps protect against tooth mineral losses from the acids produced by cariogenic bacteria [2]. Saliva also provides some buffering in the stomach [1]. Previous studies have suggested that specific feedstuffs, including certain forages and cereal based complementary feeds, may be responsible for various aspects of equine dental disease [2,3,4].

There is also a perception within the horse industry, expressed by horse owners [5], that certain forages, in particular those conserved by air exclusion and ensiling, may influence the oral cavity environment and play a role in dental disease. Forage may be conserved by drying (hay), through the exclusion of air with limited microbial fermentation (haylage), or by ensiling i.e. microbial fermentation coupled with air exclusion (silage) [6]. The main difference between haylage and silage is moisture content which is associated with the extent of fermentation; haylage typically has a higher dry matter, >50-80 %, than silage which will have a greater moisture content >50 % [6]. The combination of a low dry matter and an anaerobic environment allows for bacterial fermentation of water-soluble carbohydrate (WSC) predominantly to lactic acid (other fermentation products will also be present). Forages conserved via air exclusion with a higher dry matter content, may undergo partial fermentation or no fermentation resulting in variable WSC and pH levels in the conserved forage depending on the initial WSC content of the forage and the extent of the fermentation. Typically,

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silage has a lower pH and undergoes greater fermentation during conservation than haylage [6,7]. The low pH of silage and of some haylages, the high WSC in some hays [8] as well as the high starch content from cereal grains in some complementary feeds have been deemed to play a causal role in the formation of peripheral caries in equids [3]. These feedstuffs, may lower the pH within the mouth due to their chemical composition, for example the pH of the feedstuff itself could be acidic; or because the feed contains rapidly fermentable carbohydrates that could be fermented by the oral microbiome to produce short chain fatty acids and thereby lower the pH [9]. While these are plausible hypothesis, saliva is an alkaline solution and therefore should play a significant buffering role within the oral cavity. The original postulated link between dental caries and diet was thought to be driven by the fermentation of starch and sugar into lactate and acetate in the mouth, thereby reducing the pH in the biofilm around the tooth [10,11]. While links between specific diets and peripheral caries have been difficult to show in the UK equine population, associations have been found in other countries and certain diets such as meadow hay appear to have a protective effect against caries formation whereas oaten hay increases the risk of caries formation [8,12]. However, haylage is still being anecdotally linked with an increased risk of dental caries and therefore evidence of the effects of different feedstuffs, in particular haylages, on the pH within the oral cavity of the horse are required to understand if certain feedstuffs are likely to play a role in dental caries in equids. The aims of this study were to identify if; (a) a range of different feedstuffs and (b) four haylages with different nutrient profiles, influenced the pH of the oral cavity prior to, during and up to 30 min post feeding and the effect of differing haylage types on oral pH over a 12-day period.

2. Materials and methods

Two studies were undertaken to meet the aims of this project. Study one was a 5×5 Latin Square designed study which investigated the effect of five different feedstuffs over five days on equid oral pH prior to feeding, directly after feeding and at 15 and 30 min after feeding. Study two utilising a replicated Latin Square design of 4×4 took a more longitudinal approach using 15 day feeding periods, totalling 60 days, and focussed upon the effect of four different haylages on equid oral pH prior to and after feeding at four different time points.

Both studies were granted ethical approval by the Royal Agricultural University research ethics committee RAU161018 and RAU 20204603-Daniels.

2.1. Animals

2.1.1. Study one

Five warmblood horses aged 4-13 years (mean 8.4±4.2) were recruited onto this study. Horses were all in ridden work and were fed complementary feeds plus forage as part of their normal routine. Over the study all horses were stabled overnight and at pasture during the day at a private farm in Billingshurst, West Sussex.

2.1.2. Study two

Eight Welsh section A geldings aged 9 ± 2.5 years, mean weight 250 kg were recruited for this study. Ponies were deemed in good health from veterinary assessment at the start of the study, and had been maintained on a regular healthcare programme including parasite control and dental maintenance. During the study, ponies were housed in individual loose boxes (3.4×3.7 m) and allowed free exercise twice daily in social groups on a dry lot. Study two was conducted at the Royal Agricultural University Equestrian Centre.

2.2. Feeds

Feedstuffs selected for study one were all commercially available 1) Hay: meadow hay, 2) Haylage: a commercially produced perennial ryegrass (*Lolium perenne*) haylage, 3) Alfalfa (*Medicago sativa* L.): commercially available unmolassed alfalfa chaff with alfalfa pellets (80:20 ratio), 4) USP: unmolassed sugarbeet pulp (*Beta vulgaris*) and 5) Oats (*Avena sativa*): rolled oats. Meadow hay in study one was the primary harvest of that year (2019), the conservation data for the perennial ryegrass haylage was unknown in study one as it was commercially produced and purchased from a feed merchant rather than the producer. For study two, four commercially produced haylages were used: two haylages were mixed meadow species grasses from permanent pastures, and the other two were ryegrass haylages, rye one was Italian ryegrass (*Lolium multiflorum*) and rye two perennial ryegrass (*Lolium perenne*). Haylages were labelled by harvest date as meadow one (June 2021) and meadow two (July 2021); rye one (May 2021) and rye two (July 2021). Each haylage was a primary harvest from that season from different fields. Alongside the differences in harvest date the differences between haylages were also reflected in chemical composition (Table 1). The haylages fed in the study had been re-packed after conservation from 600 kg bales into small 20 kg bales by a commercial provider and were sourced directly from the producer (Country Haylage, Bristol, UK).

Feed pH was evaluated for each feedstuff as they would be fed. For the unmolassed sugarbeet, pH, for example, was determined from the dry pellets, as well as from the freshly soaked sugarbeet (1:5 ratio of pellets to water) and after the sugarbeet had been soaked for 24 h and stored at a maximum ambient temperature of 16°C (as it is common practice to soak sugarbeet pulp up to 24 h in advance of feeding). Feed pH was measured by adding 1:1 ratio of feed to ddH_2O into a stomacher bag and stomached (Lab-Blender 400, Worthing, UK) for five minutes to ensure the substrate and liquid were blended together. Liquid from the stomached feed was poured into a 15ml falcon tube and incubated at 16 °C, in line with maximum ambient storage temperature of the feeds, for 60 min prior to pH testing using a pH probe (Thermofisher Orion Star A21, UK). Feeds in study one were analysed for dry matter (DM), starch, water soluble carbohydrate (WSC) and ethanol soluble carbohydrate (ESC) by wet chemistry (Dairy One, USA). The haylages in study two were analysed for DM by oven drying in a forced air oven (Genlab, Cheshire, UK) at 50°C until reaching a constant weight. Haylage samples were also analysed for acid detergent fibre (ADF) [13], neutral detergent fibre (NDF) [14], crude protein (CP) [15] and WSC [16,17]. Feed nutrient compositions can be seen in Table 1.

2.3. Pilot study

Prior to the start of study one, oral pH was assessed in the morning (8 am) prior to feeding and in the evening (4 pm) prior to feeding to identify any diurnal effect on oral pH in four horses. Over three days saliva samples were collected using saliva swabs (Salimetrics®, Salivette

Table 1
chemical composition of the feedstuffs used in both studies on a dry matter basis. USP = unmolassed sugar beet pulp.

	Dry Matter g kg^{-1}	Starch g kg^{-1}	WSC g kg^{-1}	ESC g kg^{-1}	CP g kg^{-1}	ADF g kg^{-1}	NDF g kg^{-1}
Study one							
Hay	864	4	72	40	-	-	-
Haylage	736	7	67	42	-	-	-
Rolled oats	905	437	32	15	-	-	-
Alfalfa chaff	893	4	58	43	-	-	-
USP	918	85	104	80	-	-	-
Study two							
Meadow one	756	-	138	-	75	304	359
Meadow two	751	-	159	-	48	350	342
Rye one	598	-	421	-	67	196	579
Rye two	822	-	219	-	62	354	373

USA) by swabbing in the interdental space and over the tongue to collect 1 ml of saliva both am and pm. The saliva was squeezed from the swabs into the salivette collection vessel using forceps and the pH was measured immediately with a Neulog pH sensor (New York, USA). These data, analysed by paired T-test, suggested no daytime effect was present (am pH 8.62 ±0.21 pm pH 8.6 ±0.23, P=0.84), therefore samples were only collected once a day for both studies.

2.4. Study design

In study one, each horse was sampled over a five-day period and on each of the five days the horse was given a different sample of feed in order to test oral pH response (Table 2). Each of the feeds tested were already being fed to these horses on a daily basis as part of their normal ration and a 500 g sample of the desired feed was fed on its own for the purpose of testing the oral pH each day. Table 2 shows the study design for study one. The unmolassed sugarbeet pulp was soaked as per the manufacturer's instructions, soaking one-part sugarbeet nuts to five parts cold water for 12 h prior to feeding to form a pulp and stored at a maximum ambient temperature of 16°C.

Study two took a longitudinal approach, and was designed as a replicated Latin square (Table 3) whereby eight ponies were randomly paired and over four feeding periods, each lasting 15 days, they were fed each of the haylages. Periods consisted of 12 days being fed solely on a specific haylage and then a three-day change over period, days 13-15, to a different haylage. During the three-day change over the ratios of haylage from the previous feeding period and new haylage from the next period were; day 13: 70:30, day 14: 50:50 and day 15: 30:70 with 100 % of the new haylage being fed on day one of the new period. In this study haylage comprised 100 % of the diet and was fed at 1.75 % of live weight on a dry matter basis. Ponies were fed four times per day ¼ of their ration on each occasion from small-holed hay nets.

2.5. Sampling

Prior to sampling in both studies all feed and water were withheld for 30 min, baseline saliva samples were taken using saliva swabs (Salimetrics® salivettes, USA) in triplicate held in the interdental space and passed over the tongue for approximately two minutes to collect saliva. In study one sampling took place in the afternoon after horses were brought in from grazing. After baseline samples had been collected the horses were fed their 500g feed sample, and saliva samples were collected again as soon as they finished chewing the final mouthful of test feed. Further feed and water were withheld for 30 min after the test feed so that repeat samples could be collected at 15- and 30 min post prandially. All samples were collected in triplicate. Saliva was squeezed out of the swabs into salivette collection tubes using forceps to gain approximately 1ml of saliva. pH was recorded immediately post collection using a Neulog pH sensor every 0.02 seconds over a 10 second period to allow analysis to be conducted at the stables.

In study two, samples were collected in the morning from 6 am at each sampling point. The same procedure was followed for baseline samples as in study one, ponies were then fed their morning haylage feeds. After 500g had been ingested saliva swabs were taken as previously described on completion of the final mouthful of forage. Ponies were sampled on the first day of the period, six days into the period, and then again at the end of the period on day 12.

During each feeding period forage samples were collected from a freshly opened bale using a grab sample technique in a W formation from sections across the bale from each end and a central section, these three samples were then pooled for analysis to produce a composite bale sample. For each feeding period composite samples of each haylage type, representing samples from the bales fed, were produced for analysis. Upon collection saliva samples were placed on ice and after all the morning samples had been collected, they were then transported back to the laboratory within 4 h of collection. On arrival at the laboratory saliva was extracted from the swabs into collection tubes by centrifuging for 5 min at 1000 x g (Durafuge 200, Thermo Fisher, Cheshire, UK). Following extraction samples were warmed for 30 min in an incubator set to 37°C to mimic core body temperature and pH was measured using a Thermo Fisher Orion star pH meter (Massachusetts, USA) and probe on 1ml of saliva.

Table 2
Study design of experiment one, a 5×5 Latin square, for each feed on each day represented in the table.

Horse	Day 1	Day 2	Day 3	Day 4	Day 5
1	Hay	Haylage	Oats	Sugarbeet pulp	Alfalfa
2	Alfalfa	Hay	Haylage	Oats	Sugarbeet pulp
3	Sugarbeet pulp	Alfalfa	Hay	Haylage	Oats
4	Oats	Sugarbeet pulp	Alfalfa	Hay	Haylage
5	Haylage	Oats	Sugarbeet pulp	Alfalfa	Hay

Table 3
Study two design where ponies were paired to replicate the Latin square (8×4×4). Each period lasted 15 days with a 3-day change over period, days 13-15 to the next haylage diet.

Ponies	Period 1	Period 2	Period 3	Period 4
Pair 1	Rye One	Rye Two	Meadow One	Meadow Two
Pair 2	Rye Two	Meadow One	Meadow Two	Rye One
Pair 3	Meadow One	Meadow Two	Rye One	Rye Two
Pair 4	Meadow Two	Rye One	Rye Two	Meadow One

2.6. Data analysis

For both studies, data were analysed using a repeated measures ANOVA where the feedstuffs were treatments, blocked by horse/pony. Fishers least significant difference (L.S.D) was used, L.S.D = t (error degrees of freedom) x s.e.d for post-*hoc* multiple comparisons. The pH of feedstuffs was analysed by one way ANOVA using Fishers least significant difference (L.S.D), L.S.D = t (error degrees of freedom) x s.e.d. post-*hoc*.

For study two linear regression models were used to identify if the nutrient composition of the haylage influenced the oral pH. In a multiple linear model oral pH represented the Y variate and X was ADF, NDF, CP, WSC and the pH of the haylage. A second model was built to represent the soluble nutrient fractions of the haylage where Y remained oral pH and X was CP and WSC. The third model considered Y oral pH and X CP and WSC grouped by forage type. Finally, eight simple linear regression models between oral pH (Y) post feeding mean, post feeding day 1, post feeding day 6 and post feeding day 12 were constructed and grouped by haylage type against WSC (X). To be deemed a predictor of oral pH variables had to have a P value <0.05 and $R^2 \geq 0.4$. Analysis for both studies were conducted in Genstat 20th edition.

3. Results

3.1. Study one

Feed pH differed between the feedstuffs with the unmolassed sugarbeet having the lowest pH of all the diets with the dry pellets having a pH 4.45 and the 12-hour soaked sugarbeet a pH 4.5. The pH increased, ($p<0.001$) after the sugarbeet pulp had been soaked for 24 h to pH 4.66. For the other feeds pH values are shown in Table 4.

Oral pH differed between the different feeds given ($P= 0.012$, Table 4). The lowest oral pH was recorded for the saliva samples taken

Table 4
pH values of each feedstuff. The unmolassed sugarbeet (USP) was analysed both dry and after soaking. Mean oral pH values associated with each feed, irrespective of time point, are displayed with the range of values collected. Differing subscript letters between columns and within rows denote differences.

	Hay	Haylage	Oats	Alfalfa	USP dry	USP soaked 12 h	USP soaked 24 h	Probability	S.e.d.
Feed pH	6.22_{d}	7.0_{f}	6.63_{e}	5.97_{c}	4.45_{a}	4.5_{a}	4.66_{b}	<0.001	0.040
Mean oral pH	8.35_{B}	8.44_{B}	8.44_{B}	8.36_{B}	-	8.18_{A}	-	0.012	0.010
(Range)	(7.41-8.9)	(7.48-9.05)	(7.62-9.03)	(7.28-8.85)		(5.86-8.9)			

immediately post feeding of unmolassed sugarbeet pulp which was lower than oral pH for all other feedstuffs. Irrespective of feed type oral pH continued to increase at 15 and 30 min after feeding (Table 5).

When looking at the interaction over time between oral pH associated with feedstuffs and the sampling time point the response to feeding was varied, as shown in Table 5. For alfalfa and oats there was no difference in oral pH from the pre-feeding basal sample and any of the post feeding time points. For hay the pre-feeding basal pH buffered to 8.40 during ingestion and remained the same for 30 min after eating. For haylage, oral pH increased after ingestion to 8.61, returning to the basal level after 15 min but increased again after 30 min to the same pH as directly after feeding. Unmolassed sugarbeet behaved differently to all other feedstuffs by dropping oral pH from pH 8.26 at the pre-feeding baseline to pH 7.52 directly after ingestion, this was then buffered to pH 8.40 within 15 min after feeding, and was at 8.23 by 30 min after feeding (which was not different from 15 min post feeding) (Fig. 1).

The immediately post ingestion oral pH from unmolassed sugarbeet was variable between the five horses in study one and ranged from 5.98-8.35. For each of these horses the replicate oral samples were consistent therefore suggesting animal differences. Horse one had a post feeding pH of 5.98, horse two 8.12, horse three 8.35, horse four 7.59 and horse five 7.59. Table 6 below displays the delta change for each animal over the four time points in study one for USP.

3.2. Study two

Comparing the pH of each of the haylages themselves as shown in Table 7, the pH of Rye one was lower than all others. Meadow two was not different from Rye two, and both of these haylages had a higher pH than Meadow one and Rye one. While these haylages differed in pH, botanical composition and plant maturity, biologically in terms of conserved forage, there was little difference in the pH between the four haylages.

There were differences in oral pH when feeding the different haylage types ($P<0.002$) as shown in Table 7. The difference in oral pH over the period was attributed to haylage type, as feeding the ryegrass haylages led to a lower mean oral pH (8.29 -8.30) over the period compared to feeding the meadow grass haylages (8.39-8.40). There was no difference in the pre-feeding oral pH for the haylages over the feeding periods, but directly after feeding the mean pH for the period was lower for rye haylages than meadow two, whereas meadow one oral pH was not different from any of the other haylages (Table 7).

There was an effect of time on oral pH in both the pre feeding saliva samples and post feeding saliva samples but there was no interaction between time point and haylage type when considering just pre-feeding or post-feeding oral pH. Pre-feeding oral pH was higher on day 1 of the period (8.26) and then dropped by day 6 (8.19) and remained the same at day 12 (8.19). Post feeding oral pH did not differ between day 1 (8.39) and day 6 (8.42) but had increased by day 12 (8.48) (Table 8 The mean values of oral pH associated with each haylage diet can be seen in Table 8 for both pre-feeding and post feeding.

Fig. 2 shows that typically the rye haylages had a lower pre-feeding pH than the meadow haylages especially on days 6 and 12. For each of the haylages at each time point post eating the oral pH increased compared to pre-feeding.

Basal, pre-feeding, oral pH fluctuated over the feeding period between pH 8.1 – 8.4 at each sampling point. Oral pH post eating, irrespective of haylage type, remained the same or was buffered to pH 8.3-8.5 directly post feeding. Ponies had the highest basal oral pH when fed meadow two at the beginning of a period and by day 12 the basal oral pH was lowest when fed either of the rye haylages compared to the meadow haylages.

When looking at the nutrient profile of haylage to identify if this could be used as a predictor of post feeding oral pH three multivariate linear regression models were applied. The first model considered ADF, NDF, CP, WSC and the pH of the forage. However, none of these nutrients could predict oral pH (R^2 0.10, P= 0.173). The second model only considered the soluble nutrients CP and WSC as predictors of oral pH, and again neither of these nutrients could predict oral pH (R^2 0.10,P= 0.387). The final model considered the soluble constituents, crude protein and WSC, grouped by haylage (R^2 0.11, P=0.715). These models were repeated for pre-feeding oral pH, the first model considering ADF, NDF, CP, WSC and the pH of the forage could not predict the mean pre feeding oral pH (R^2 0.16, P=0.383). The second model considered WSC and CP (R^2 0.085, P=0.580) and the final multiple regression model considered WSC and CP by haylage type (R^2 0.07, P=0.743).

Simple linear regression between mean post feeding oral pH across all three time points and WSC grouped by haylage type could not be used to predict oral pH of any haylage type (R^2 0.04, P=0.578). From the regression model for day 1, WSC was unable to predict post feeding oral pH for any of the haylage types (R^2 0.00, P=0.844). For day 6 the model could not predict post feeding oral pH when grouped by haylage type (R^2

Table 5
Oral pH over time and the interaction between oral pH on each feed over each of the time points within study one. Differing subscript letters next to means within a row denote differences over time. USP= unmolassed sugar beetpulp.

	Pre-feeding (basal)	Directly post feeding	15 min post feeding	30 min post feeding	Significance	S.e.d.
Mean Oral pH	8.31_{A}	8.28_{A}	8.41_{B}	8.42_{B}	0.025	0.010
(Range)	(7.28-8.89)	(5.86-9.05)	(7.43-8.9)	(7.76-9.03)		
Hay oral pH	8.12_{a}	8.40_{b}	8.48_{b}	8.42_{b}	<0.001	0.130
(Range)	(7.41-8.89)	(8.13-8.69)	(7.75-8.86)	(7.76-8.9)		
Haylage oral pH	8.30_{a}	8.61_{b}	8.27_{a}	8.58_{b}		
(Range)	(7.48-8.83)	(8.1-9.05)	(7.84-8.89)	(8.04-8.97)		
Oats oral pH	8.29_{a}	8.51_{a}	8.52_{a}	8.43_{a}		
(Range)	(7.71-8.61)	(7.62-8.9)	(8.27-8.73)	(8.01-9.03)		
Alfalfa oral pH	8.31_{a}	8.35_{a}	8.38_{a}	8.44_{a}		
(Range)	(7.28-8.83)	(7.67-8.71)	(7.43-8.85)	(7.96-8.84)		
USP (soaked 12 hrs) oral pH	8.26_{b}	7.53_{a}	8.40_{b}	8.23_{b}		
(Range)	(8.18-8.76)	(5.86-8.37)	(7.88-8.9)	(7.9-8.5)		

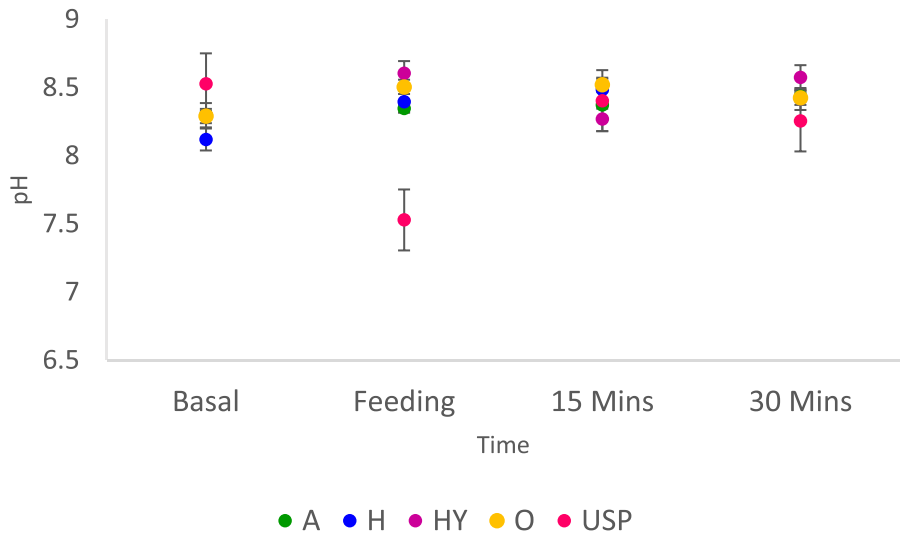

Fig. 1. Oral pH response to different feeds; A: Alfalfa chaff with alfalfa pellets, H: meadow hay, HY: ryegrass haylage, O: rolled oats and USP: unmolassed sugarbeet. pH over time; prior to, post ingestion and at 15- and 30-minutes post ingestion, $P < 0.001$, errors bars represent standard error.

Table 6
Delta change in oral pH for each of the horses in study one when fed Unmolassed sugar beet pulp (USP). The difference between basal pH and directly post ingestion, then the difference between directly post ingestion and 15 min and finally the difference between 15- and 30-minutes post ingestion. These data demonstrate the individual animal response to ingesting USP showing the range over the five horses over time.

Horse	Directly post ingestion of USP, pH change	15 min post ingestion of USP, pH change	30 min post ingestion of USP, pH change
1	-2.51	+2.62	-0.60
2	-0.17	+0.24	-0.11
3	-0.17	-0.35	+0.37
4	-1.06	+0.93	-0.20
5	-1.07	+0.93	-0.20

Table 7
pH of each of the haylages in study two and the mean oral pH of ponies over all time points, pre feeding and post feeding when fed each of the haylages. Subscript letters within rows denote differences.

	Meadow one	Meadow two	Rye one	Rye two	P Value	S.e.d
pH Haylage	5.80_{b}	5.92_{bc}	5.67_{a}	5.96_{c}	<0.001	0.063
Mean oral pH over all time points	8.38_{b}	8.40_{b}	8.29_{a}	8.30_{a}	<0.002	0.033
Mean oral pH pre-feeding of all time points	8.33	8.22	8.18	8.12	0.082	0.080
Mean oral pH post feeding for all time points	8.45_{ab}	8.48_{b}	8.39_{a}	8.40_{a}	0.020	0.030

0.03, P=0.378). Finally for day 12 oral pH could not be predicted by WSC by diet (R^2 0.03, P=0.550). Collectively these data suggest that WSC alone or a broader nutrient profile were not a good predictor of post feeding oral pH.

Simple linear regression models were also built for pre-feeding oral pH over the feeding time points regressed against WSC for each diet type. The initial model considered mean oral pH over the feeding period which could not be predicted by WSC (R^2 0.03, P=0.731). For day 1 of the feeding period WSC could not predict pre-feeding oral pH (R^2 0.08, P=0.784). By day 6 WSC could predict pre-feeding oral pH (R^2 -0.4, P=0.022) specifically for rye one (R^2 0.85, P=0.045). By day 12 there was a weak relationship where WSC could predict pre-feeding oral pH (R^2 -0.33, P=0.035) however this could not be attributed to any specific haylage diet.

Table 8
Effect of sampling time point on oral pH for all study periods and all haylage types. Subscript letters within rows denote differences.

	Day 1	Day 6	Day 12	P value	S.e.d.
Pre feeding (combined)	8.26_{a}	8.15_{b}	8.19_{b}	0.026	0.030
M1	8.23	8.22	8.21		
M2	8.42	8.24	8.33	0.288	0.095
R1	8.24	8.19	8.12		
R2	8.15	8.10	8.11		
Post ingestion (combined)	8.39_{a}	8.42_{a}	8.48_{b}	0.008	0.030
M1	8.36	8.47	8.53		
M2	8.39	8.48	8.55		
R1	8.38	8.33	8.45	0.180	0.059
R2	8.42	8.38	8.39		

4. Discussion

Collectively the findings from these studies suggest that feedstuffs can influence oral pH directly post ingestion. Predominantly oral pH increased after feeding and in study two the post feeding pH was always higher than the basal pH at each time point measured. Given that the haylage pH ranged from pH 5.67 – 5.96 this suggests that the saliva produced had provided ample buffering even after 30 min of continuous eating of the morning ration. However, in study one, the oral pH did decrease following the feeding of USP although the oral pH had returned to baseline within 15 min of feeding. Oral pH remained alkali at all times in both studies, with the exception of the provision of USP when the pH lowered directly after ingestion in some individuals.

The results in study one demonstrated that the feeds that may have been expected to lower oral pH, i.e., oats and haylage, based upon previous studies that considered complementary feeds and silage [3], gave the two most alkali readings directly after feeding. Previous studies as mentioned before, have suggested that ensiled and air excluded forages are associated with dental disease in horses [3,4], and this is also a common conception from equine dental technicians (personal discussions). In study one the pre-feeding oral pH on hay was lower than prior to and post feeding haylage. It is important to note that the pH of the

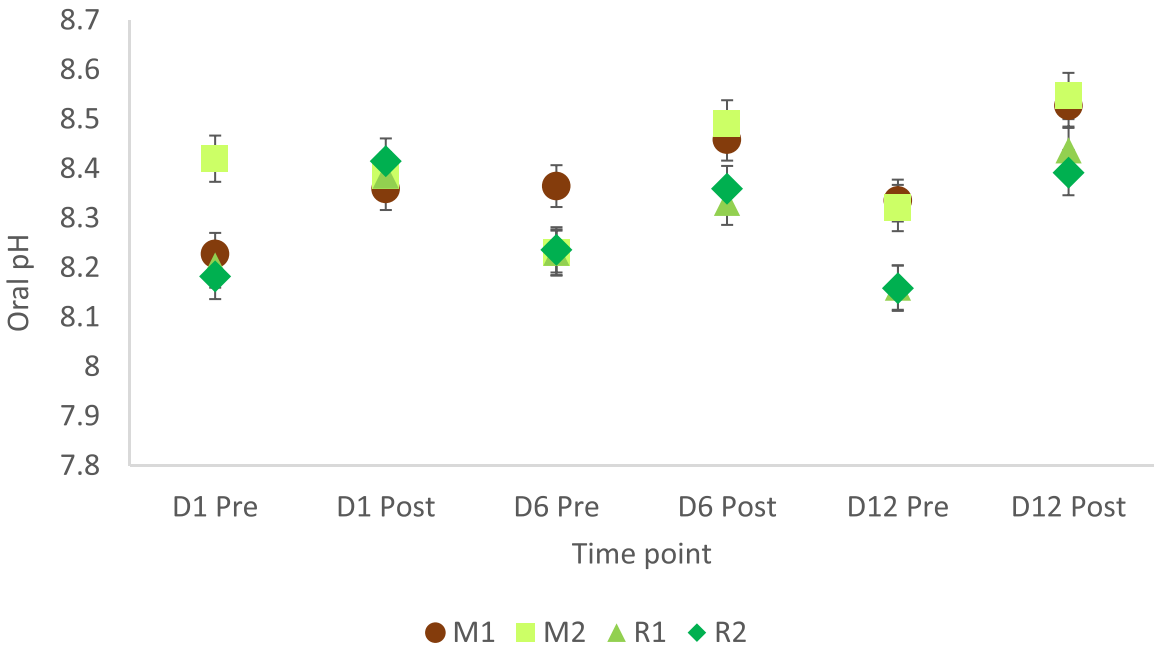

Fig. 2. Basal oral pH (pre) and oral pH after feeding 500g/1kg DM (post) of each haylage type at each of the time points. Haylages types; M1 Meadow one, M2 Meadow two, R1 Rye one (Italian) and R2 Rye two (Perennial) denoting early and later cut haylage of each haylage type. Error bars represent standard error.

haylage itself in study 1 was neutral but in study 2 all the haylages had an acidic pH. The pH of haylage will be influenced by the preservation process depending on the moisture content which determines if fermentation has occurred, by converting more WSC into lactic acid thus providing an end product with a low pH [6,7]. Commercially produced ryegrass haylage was selected in study one as this forage was produced from a monoculture known to commonly have high WSC content. From the analysis the moisture content of the haylage was 26.4 % and therefore this may be deemed as a dryer haylage which would have been a limiting factor for conservation by fermentation. In study two the rye grass haylages had higher WSC contents, rye one: 421 g kg^{-1} and rye two: 219 g kg^{-1} compared to the haylage in study one. The moisture content in rye one, ~40 %, would suggest it was plausible that some fermentation could have occurred during conservation, however the WSC content remained very high. The haylage was not evaluated for fermentation products in this study so no direct conclusions can be drawn from moisture, pH and WSC data alone. Despite the lower pH of these haylages neither of them resulted in a neutral or acidic oral pH at any point during study two and while there were differences in the pH between the haylage types none of these were found to be biologically relevant to oral pH. Gere and Dixon [3] proposed that the occurrence of peripheral caries in their study was likely due to the feeding of high moisture haylage and cereal based concentrate feeds. However, their study was a post-mortem study and there was no information provided of the diet that these horses were fed. The hypothesis of Gere and Dixon [3] was based upon previous studies [18,19] when prior to the widespread feeding of haylage in Sweden the prevalence of peripheral caries in 335 Swedish horses was only 0.9 %. In comparison Gere and Dixon [3] reported 6.1 % prevalence from 510 horses following the increased use of ensiled forage feeding in Sweden, however this study lacked data on the diets of horses in the study and therefore this figure should be interpreted with caution. A more recent study from Borkent and Dixon [4] reported that haylage and silage could not be identified as a risk factor for peripheral caries in horses and our present study would support that, given that low oral pH was not observed around the time of feeding. In study one oats were selected as being a single source of starch, and although the oats contained 395 g kg^{-1} of starch and 32 g kg^{-1} of WSC this did not appear to influence the oral pH at the time of feeding. Starch is not water soluble and equid saliva contains very low concentrations of α-amylase, (0.44 U/ml when compared to 0.77 U/ml in human saliva [20]) meaning that little may have been available for bacterial fermentation during the short time present in the mouth. This, therefore, could provide a potential explanation for the lack of effect of the rolled oats on oral pH. This hypothesis may also apply to the haylages in study 2, where rye one and rye two contained more WSC than the meadow haylages yet this did not consistently influence the oral pH. It is plausible that the amount of chewing (and mixing with the alkaline saliva) required to form a bolus from the haylage would have diluted any WSC released into the oral cavity for fermentation at the time of ingestion. The composition of WSC includes a variety of sugars and it is likely that these will differ between the differing plant species within each haylage. A haylage with WSC with a cytosolic high sucrose or glucose content that could become available at least in part on chewing would have readily available substrate for bacterial fermentation. Whereas WSC with a high fructan content potentially would not be as readily available to the oral microbiome especially if due to the structure of the forage it was not released as readily post chewing or there was less chewing of that haylage type due to its chemical composition. It is plausible, therefore, that the botanical differences between the haylage types may explain the inconsistent effect of WSC on oral pH. In these studies, WSC composition was not analysed so it is not possible from these data to form strong conclusions on the effect of WSC composition on oral pH but this does provide a plausible explanation for the inconsistent WSC response with haylage type. In study two we did observe a relationship between pre-feeding oral pH and WSC specifically when feeding rye one after six days of the feeding. However, this finding suggests that as WSC increased so did oral pH, whereas for the other three haylages as WSC increased pre-feeding oral pH decreased. By day 12 of our feeding periods M2, R1 and R2 all demonstrated positive correlations as oral pH increased so did WSC, whereas M1 showed a negative relationship, where oral pH decreased as WSC increased. These findings may be a reflection of differing compositions of WSC within these haylages. Water soluble carbohydrate in forage may influence oral pH outside of the feeding period which may be attributed to fermentation in the oral microbiome, given the structure of the bacterial community profile that has previously been reported [26]. In study 2 we observed a change in the pre-feeding oral pH at day six and twelve after introducing the forage, however, at all times oral pH remained alkali. If

lower oral pH were a reflection of bacterial fermentation of any remaining WSC in the oral cavity post ingestion then this effect would likely be seen over a longer period of time rather than directly after feeding which was the objective of these present studies. Evaluating post prandial oral pH for several hours after feeding would therefore be interesting to explore. It may also be that a longer time period being fed on the diets may be required to assess the longer-term impacts of different feeds on oral pH both around the feeding event and in particular on basal oral pH prior to feeding.

The most interesting result perhaps came from the unmolassed sugarbeet which was the feed with the lowest feed associated pH and the only feedstuff that reduced oral pH immediately post feeding. Sugarbeet is normally fed to horses soaked to minimise the likelihood of 'choke' which can occur if the feed swells when mixing with saliva following chewing. The soaked sugarbeet is often left to absorb water for 24 h before feeding. Interestingly the pH of the sugarbeet increased during this 24 h soaking, in our study suggesting that it was not being fermented during soaking. This study was conducted over the winter months and therefore the soaked sugarbeet was stored at low ambient temperature, maximum 16 °C. Any lack of fermentation, therefore, may not be the case if left to soak at higher environmental temperatures. Considering that sugarbeet is a good source of soluble fibre [21] the pH of this feed source was lower than expected. The low pH of the processed sugarbeet pellets appears to be linked to the processing of this feedstuff and is normal for this substrate [22]. It is also possible that the low starting feed pH effect was coupled with potential fermentation of the highly soluble residual WSC content (100 g kg^{-1} DM) in the mouth, however, given the oral pH returned to basal level within 15 min of feeding it suggests that it is unlikely that fermentation occurred in this short time.

One other possible explanation for the reduced oral pH immediately following feeding unmolassed sugarbeet pulp could be the reduced amount of chewing required to form a bolus. Meyer et al. [23] reported that horses produced saliva in quantities of 4.6-6.5L/Kg/DM for haylage and hay, whereas they only produced 2.3L/Kg/DM for sugarbeet pulp. The soaked beet pulp would potentially have an even smaller particle size that would require even less chewing to form a bolus. Given that equids only produce saliva when chewing, the soft consistency of the pulp would require few jaw movements to form a bolus so the combination of the lower pH of the substrate, readily available WSC and less saliva production could explain the lower oral pH at the time of feeding. This theory may also explain why the pH in the oral cavity increased post feeding the haylages at all times in study two. Unlike the USP that required few jaw movements for bolus formation the number of jaw movements in the ponies to chew the haylage would have led to significant saliva production, rather than a readily formed bolus with the soaked USP. The increased amount of saliva produced to chew the haylage and form a bolus would act to buffer the pH of the forage in the mouth, and the results would suggest that this was capable of buffering the pH of all of the haylages in the study.

When considering the nutrient profiles of the haylages in study two, the rye grass haylages led to a lower oral mean pH over the feeding periods than the meadow grasses. While both of the ryegrass haylages contained more WSC than the meadow haylages, rye one contained 200 g kg^{-1} of WSC more than rye two, yet this did not seem to influence oral pH at any time point. The regression models in study two suggest that the individual nutrient content and WSC of the haylage alone was not a reliable predictor of oral pH. Differing WSC profiles from differing grass species, alongside other environmental factors, may vary in consistency which may be a reason that WSC was not a good predictor of oral pH. Given that Gere and Dixon [3] and Borkent and Dixon [4] suggested that high moisture conserved forages and high sugar diets were more likely to lead to dental caries, the data in our present study in contrast would suggest that such feedstuffs have limited effect on oral pH which has previously been indicted in playing a role in caries formation [3,4]. Study two took a longitudinal approach, when compared to study one, with 12-day periods on each haylage where the haylage made up 100 % of the diet. Rye one with the lowest haylage pH and highest WSC content of the forages had little effect on the resting oral pH. However, when looking at the four haylages, the meadow haylages, which had the lower WSC contents did tend to have a higher basal pH over the feeding periods than the ryegrass haylages with their greater levels of WSC. Jackson et al. [8] identified that meadow hay was protective against caries formation and oaten hay was associated with peripheral caries formation. It is possible that forages with lower fermentable sugar content and with more structural fibre requiring more chewing provide less substrate for plaque bacteria and result in more saliva being produced as a buffer, which may have a protective effect against caries formation. However, horses have also been seen to extensively chew higher moisture forage e.g., soaked hay compared with dry hay [24], which might also explain why in study two there was no change in oral pH post feeding samples even following intake of rye one, with its low pH and high WSC, most likely due to the amount of chewing involved with its ingestion leading to sufficient saliva production to act as a buffer.

Lundstrom et al. [12] identified that the composition of equid saliva after chewing various types of preserved forages (hay, haylage and silage), did not differ. They also noted hay can have a pH similar to haylage which they suggested could partially explain this. However, it is likely that the complete nutrient profile of the forage will influence oral pH as discussed above. Equid saliva has a higher concentration of bicarbonate compared to human saliva [12] and this may also influence the extent of the buffering seen even with low pH feedstuffs as in our studies. However, it is important to note that in both of our present studies the pH of the orally collected saliva was the only measure taken. The pH at the gingival level was not recorded and this area may be more reflective of the relationship with dental caries than saliva, which is a limitation of these studies. The pH of plaque was recorded by Lundstrom et al. [12] in their study which dropped significantly when sucrose solution was applied to teeth with caries but did not drop when sucrose was applied to healthy teeth. Lundstrom et al. [12] highlighted that equine peripheral caries occur in an oral environment somewhere between pH 5.7-6.7 but the critical value for equine cementum is unknown. When considering the oral pH in our present study the only feedstuff to bring the oral pH down to this level was unmolassed sugarbeet pulp. It is, therefore, plausible that dental caries are a multifactorial disease in which diet may play a role [2]. However, it is clearly more complicated than just the presence of low pH feedstuffs in the mouth. From study one, although the oral pH was quickly buffered following the ingestion of unmolassed sugarbeet the feeding of this substrate to horses with dental caries warrants further investigation. Horse one in study one had a lower oral pH consistently after sugarbeet than the other four horses. It is plausible that horse one may have had some underlying/undiagnosed dental caries and the reduced oral pH in horse one was the result of the presence of soluble sugar around those teeth as previously reported [12]. At the time of the study all horses to the best of the authors knowledge had had regular dental checks with no reports of peripheral caries. However, it does appear that in study one horse one responded more to the sugarbeet directly after ingestion than the other horses in the study. This could potentially reflect individual susceptibility to diet induced peripheral caries or enhanced changes in the oral cavity.

These present studies were focused upon the effect of feed substrates on oral pH, but as mentioned above it is likely that bacteria in the oral microbiome play a role in the formation of dental caries. Bacteria have been reported to enter the peripheral cementum as part of the pathological changes to the affected teeth [25]. Recent studies have also identified *streptococcus, lactobacillales, veilonella* and *Acitinomycetoa* present in the oral microbiome of equids with peripheral caries as opposed to healthy controls [26]. While there has been some work to identify the bacterial community profile of hays [27,28] no such work has been conducted on haylage. However, when looking at the bacterial community profile of hays, bacteria associated with formation of equid

dental caries can be found on such forages [28]. Importantly, these bacteria can be successfully eliminated from the forage by high temperature steaming prior to feeding [28]. More work is required to identify whether bacteria associated with dental caries are present on ensiled or air excluded forages in the same way that they are present on hays and how relevant they are to the formation and/or persistence of dental caries.

A limitation in these studies was the immediate post prandial saliva samples were collected directly after eating and there is a possibility that swabs could have been contaminated with residual feedstuffs, as mouths were not washed-out following feeding as this may also have influenced the oral environment directly after washing. It should also be noted there were differences between studies in the measuring of the oral pH. In study one this was undertaken on site with a mobile pH meter, whereas in study two samples were collected on ice and analysis was undertaken in the laboratory. This methodological difference should be considered when interpreting the data and drawing direct comparisons between studies one and two. The focus of study one was on the oral pH around the time of feeding over a short time period, and in this study, horses remained at pasture during the day which may have influenced the oral microbiome. This was addressed in study two where ponies had no access to pasture and the haylage formed the sole ration. Finally, both of these studies were conducted over a short time period focusing on the oral pH around the time of feeding using small groups but achieving statistical power. The association between diet and peripheral caries is likely to be linked to the oral microbiome and fermentation of substrate rather than purely the acidity of the feed. To truly test this hypothesis based upon previous case studies [2], longer feeding periods up to six months would be required to determine if feeds altered the basal oral pH over time after introducing the feeds. Notwithstanding the limitations of these two studies, we believe that these data provide a valuable insight into the effect of various feedstuffs on the oral pH of horses around the time of feeding. While feedstuffs themselves can be acidic, the buffering capacity of saliva prevented reduction of oral pH to an acidic level at least orally. Initial findings suggest that these diets alone do not appear to alter the oral pH of horses, but more work is warranted into the effect of substrates such as unmolassed sugarbeet and very high sugar feeds especially in horses with dental caries.

5. Conclusion

It is possible for feedstuffs to temporarily alter oral pH in horses, however, in most cases the effect of chewing, with its associated saliva production, buffers oral pH around the time of feeding. Following ingestion of unmolassed sugarbeet, there was a transient decrease in oral pH in some individuals. The feedstuff itself has a low pH and in its soaked state requires minimal chewing for bolus formation, and therefore the low oral pH post feeding potentially was due to a combination of acidic substrate and limited saliva production. However, buffering back to the basal alkaline pH occurred within 15 min of eating. Other feedstuffs did not demonstrate this reduced oral pH post feeding. From study two it would appear only WSC in the haylage could be attributed to the changes in pre-feeding oral pH over time, however no specific nutrient altered post-feeding oral pH. It, therefore, appears that the chewing associated with feeding ensures that saliva remains above pH 7 even when feeding acidic forages. These data, however, only reflect the effect on oral pH rather than the effect of substrate core pH or ingestion on gingival level pH which may differ. In addition, these studies did not consider bacteria in the mouth or on the feedstuffs which may have a closer association to the development of dental caries.

Data Statement

Data are available from the corresponding author upon request.

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Ethical statement

Both studies were granted ethical approval by the Royal Agricultural University research ethics committee RAU161018 and RAU 20204603-Daniels.

CRediT authorship contribution statement

S.P. Daniels: Writing – original draft, Supervision, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **E.J. Whiteside:** Investigation, Data curation. **S. Martin:** Formal analysis, Conceptualization. **M.J. S. Moore-Colyer:** Writing – review & editing, Data curation. **P. Harris:** Writing – review & editing, Conceptualization.

Declaration of competing interest

There are no conflicts of interest to declare.

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