Production of amines in equine cecal contents in an in vitro model of carbohydrate overload

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ABSTRACT: Acute laminitis can be induced experimentally in horses by the administration of carbohydrate, resulting in fermentation within the cecum and ischemia-reperfusion of the digits. The products of fermentation that trigger acute laminitis are as yet unknown; however, compounds such as amines might play a role due to their potential vasoactive properties. The objectives of this study were to quantify the amines present in equine cecal contents and to use a model of carbohydrate overload in vitro to test the hypothesis that carbohydrate fermentation is associated with increased amine production. Cecal contents from each horse were divided into aliquots and incubated anaerobically with either cornstarch or inulin (a form of fructan carbohydrate; both 1 g/100 mL). The pH was measured and samples were taken at the same time for amine measurement by HPLC at 2-h intervals over a 24-h period. In a second set of experiments, the effects of the antibiotic virginiamycin (1 mg/100 mL), calcium (CaPO₄; 0.3 g/100 mL), and plant steroidal saponin (Yucca schidigera extract; 0.1 g/100 mL) were examined on pH and amine concentrations in cecal contents incubated with starch or inulin. Both starch and inulin caused significant time-dependent falls in pH, from 6.7 ± 0.1 at 0 h to 5.2 ± 0.1 (starch) and 5.0 ± 0.1 (inulin) at 24 h. Fermentation of carbohydrate was also associated with increased production of phenylethylamine and isoamylamine (two- to threefold increases) as well as putrescine and cadaverine (1.5- to twofold increases). Virginiamycin inhibited the fall in pH and increases in production of phenylethylamine and isoamylamine, while calcium phosphate moderated the changes in pH only. Yucca schidigera extract was without effect. These data show that fermentation of carbohydrate by equine cecal microbiota may lead to increased production of amines.

Key Words: Amines, Carbohydrate Loading, Cecum, Equidae, Laminitis

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Introduction

Fermentation of carbohydrate in the hindgut has been recognized as one of the primary events leading to acute laminitis in the horse (Garner et al., 1978). Dietary starch, if present in excess (>0.4% body weight; Potter et al., 1992), may be fermented by cecal and colonic Gram-positive bacteria, producing lactic acid. Fructans, the storage oligosaccharides present in grass, do not appear to be digested by the equine small intestine and so may also act as substrates for fermentative bacteria.

The factor(s) released from the hindgut as a consequence of fermentation, that trigger the lamellar ischaemia thought to lead to the clinical syndrome of acute laminitis (Hood et al., 1993) have not yet been elucidated. Amine compounds, produced by the decarboxylation of amino acids by various bacteria (Rice and Koehler, 1976), may cause peripheral vasoconstrictor effects by virtue of their structural similarities with endogenous vasoconstrictor amines, such as serotonin and catecholamines. Indeed, amines have been shown to cause the constriction of isolated digital blood vessels in vitro (Bailey et al., 2001).

Virginiamycin, a streptogrammin antibiotic, has been used to prevent starch overload-induced laminitis by preventing the overgrowth of Gram-positive bacteria (Rowe et al., 1994). Another product that may inhibit the growth of one of these bacteria, Streptococcus bovis, is steroidal saponin, of which the extract of the desert plant Yucca schidigera is a good source (Wang et al., 2000). Inorganic calcium salts such as CaHPO₄ have been shown to moderate the fall in pH associated with the fermentation of fructans in the rat cecum (Remesy et al., 1993), but have not been studied in the horse.
This study identifies the amines in equine cecal contents using an HPLC method, and measures their production in an in vitro model of carbohydrate overload. We examined the effects of virginiamycin, CaHPO₄ and *Yucca schidigera* extract on pH and amine production in this model system.

**Materials and Methods**

**Anaerobic Incubation of Cecal Contents**

Horses that had been fed predominantly hay over the preceding 1 to 2 wk were killed at an abattoir and were used as the source of the cecal contents for this study. Approximately 1 L of cecal contents was collected into sterile containers flushed with nitrogen gas, with care being taken to preserve anaerobic conditions. The contents were chilled to approximately 4°C for transport to the laboratory. All of the incubation experiments were carried out under anaerobic conditions, at 37°C (Mk 3 anaerobic work station, Don Whitley Scientific Ltd., Basingstoke, UK).

The cecal contents from individual horses were strained to remove coarse fibrous material (over 0.5 cm in length), and then divided into 100-mL aliquots, with no subsequent dilution. In the first set of experiments, involving cecal contents from 10 horses, either corn-starch (1 g/100 mL) or inulin (1 g/100 mL) were added to one aliquot from each animal, with another aliquot incubated without added carbohydrate to act as a control. Cecal contents were incubated for 24 h at 37°C while being agitated (Orbital shaker, Jencons-PLS Ltd., Leighton Buzzard, Bedfordshire, UK), and during this time the pH of the fluid in each aliquot was measured at 2-h intervals (model 3051 pH meter, Jencons-PLS Ltd., Leighton Buzzard, Bedfordshire, UK). Samples (1 mL) were also taken of the fluid at these time points for determination of amine concentration by HPLC. Preliminary concentration studies were undertaken to examine the effects of various carbohydrate concentrations (0.25 to 5 g/100 mL) on cecal fermentation as measured by changes in pH, and 1 g/100 mL of both starch and inulin was found to be sufficient to produce maximal effects.

In a second set of experiments, virginiamycin (1 mg/100 mL), calcium hydrogen phosphate (CaHPO₄; 0.3 g/100 mL), or *Yucca schidigera* extract (0.1 g/100 mL) were added to aliquots of cecal contents to which either starch or inulin had been added (both 1 g/100 mL). Positive controls, containing 1 g/100 mL of starch or inulin, and a negative control, with no added carbohydrate, were also run. The cecal contents were incubated for 24 h and pH measurements and samples for amine determination were taken at 2-h intervals as before. Cecal contents taken from four animals were used in these experiments, and data referring to the 0- and 24-h time points were expressed as the mean ± SEM.

**HPLC Analysis of Amines in Cecal Contents**

The HPLC methods used in the present study were adapted from those described by Busto et al. (1994) and Buiatti et al. (1995) to analyse amines in wines and beers, respectively.

The samples of equine cecal contents were centrifuged at 1,500 × g for 15 min at 4°C, and the supernate was filtered (Whatman no. 1 filter paper). Aliquots were frozen and stored at −80°C until required for assay. One milliliter of cecal contents was diluted 1:4 vol/vol with acetone:water (2:1), and the resulting solution was made basic by the addition of 1 mL of borax buffer (3.81 g sodium tetraborate in 100 mL of distilled water adjusted to pH 10.5 with 10 M sodium hydroxide). The internal standard heptylamine was then added to give a final concentration of 5 µg/mL. The amines were derivatized with 1% dansyl chloride in acetone at 65°C for 25 min and then extracted using SEP-PAK C18 solid-phase extraction cartridges (6 mL, 500 mg; Waters Ltd., Watford, Hertfordshire, UK).

Separation was carried out using a Waters liquid chromatograph (Waters Ltd.) comprising a model 600E gradient controller and pump, a model 484 UV absorbance detector, and Waters model 746 integrating data module. The injection of the samples was carried out by means of a Shimadzu model SCL-6A autoinjector. Separation was carried out using a Waters Symmetry Shield RP_{18} column (150 × 3.9 mm i.d., particle size 5 µm) preceded by a guard column (Waters Symmetry Shield RP_{18}, 20 × 3.9 mm, 5 µm). Compounds were detected by measuring absorbance at 250 nm.

Amine compounds were identified and quantitated from standard curves constructed from the pure compounds. Intra- and interassay coefficients of variation were calculated from the standard deviation of the mean of five determinations of standards at concentrations of 1, 5, and 25 µg/mL, measured either within or between assay runs, and from five determinations of amine concentration in cecal contents, expressed as a percentage of the mean value. The assay sensitivity for each amine was calculated from the extrapolation of a dilution curve, taken as the lowest concentration giving a signal-to-noise ratio of 3:1 (Snyder et al., 1997).

**Statistical Analyses**

All curve fitting and statistical comparisons were performed using GraphPad Prism version 3.00 for Windows, GraphPad Software, San Diego, CA.

All data were expressed as mean ± SEM. The effects of the two carbohydrate sources on the pH and amine concentrations were compared to the controls by means of two-way (repeated measures) analysis of variance followed by Bonferroni’s post hoc test. The effects of the inhibitors, virginiamycin, calcium phosphate, and *Yucca schidigera* extract, on pH and amine concentration were assessed at the 24-h time point comparing the results obtained in the presence of the inhibitors to the positive and negative controls using one-way analysis of variance with Fisher’s multiple comparison. In all cases, significance was accepted at *P* < 0.05.
Results

Assay Validation

Good recovery and separation of all the amines was achieved using the extraction and chromatographic conditions described. The intraassay coefficients of variation in cecal samples were between 3.26 and 7.24% and the percentage recoveries of the standard amines added to cecal contents ranged from 87.7 to 100.7%. The detection limits for each amine varied between 0.03 and 0.22 μg/mL.

Identification of Amines in Equine Cecal Contents

Fifteen amines were identified in equine cecal contents, according to their chromatographic retention times, at concentrations greater than 1 μM. They were as follows (all concentrations are expressed as micromolar values): the aliphatic monoamines methylamine (195.5 ± 14.4), ethylamine (50.3 ± 42.5), propylamine (17.3 ± 11.2), isoamylamine (15.7 ± 1.5), and isobutylamine (5.3 ± 5.2); the aromatic monoamines tryptamine (7.0 ± 6.4), tyramine (3.7 ± 1.3), kynuramine (5.5 ± 3.4), and phenylethylamine (16.7 ± 8.9); and the diamines putrescine (23.4 ± 8.5), cadaverine (12.7 ± 3.9), histamine (1.4 ± 0.8), diaminoheptane (19.7 ± 3.9), spermidine (12.2 ± 2.3), and spermine (2.9 ± 2.8).

Effect of Carbohydrate on pH and Amine Concentration

The addition of starch or inulin resulted in a significant decrease in pH over the 24-h period of incubation (Figure 1), of 1.5 ± 0.2 (starch) or 1.7 ± 0.3 (inulin) pH units. Inulin caused a more rapid pH fall than starch, the decrease becoming significant within 6 h compared to 12 h for starch.

The concentration of four amines was significantly increased after 24-h incubation with added carbohydrate (both starch and inulin). Phenylethylamine and isoamylamine showed increases in concentration over time that mimicked the changes in pH (Figure 2a and b). A significant increase in phenylethylamine concentration was detected after 10 h of incubation with starch and 16 h incubation with inulin. For isoamylamine, a similar pattern was observed. Increases in putrescine and cadaverine were delayed until 18 and 20 h of incubation, respectively, and appeared to occur sooner and were more marked when starch was the substrate provided for the cecal cultures (Figure 2c and d). The concentration of diaminoheptane significantly increased with respect to time; however, this change was also noted in the absence of added carbohydrate.

Effect of Inhibitors

Virginiamycin (1 mg/100 mL) significantly inhibited the fall in pH resulting from added carbohydrate after 24 h, almost completely in the case of starch (Table 1). Virginiamycin also significantly inhibited the increase in phenylethylamine concentration induced by starch and inulin (positive control) by 40.6 and 24.0% respectively. The percentage inhibition of isoamylamine formation by virginiamycin was 42.3 and 32.0% for starch and inulin respectively. Similarly, virginiamycin inhibited the rise in putrescine concentration induced by starch by 69.8% but had no significant effect on the change in putrescine concentration induced by inulin. Changes in cadaverine concentration induced by starch or inulin were not affected by virginiamycin.

Calcium hydrogen phosphate (0.3 g/100 mL) also partially prevented the drop in pH associated with carbohydrate overload but had no significant effect on amine production, apart from a partial inhibition of phenylethylamine production in the presence of inulin (12.8%). Yucca schidigera extract (0.1 g/100 mL) had no significant effects on pH change or on the concentration of...
Amine production in carbohydrate overload

Discussion

These data demonstrate that a wide range of amines are present at relatively high concentrations within the cecal contents of horses and, furthermore, that the concentrations of a number of these compounds increase in conditions mimicking carbohydrate overload.

The 15 amines identified in the present study have previously been found in samples of wines, beers, and fermented foodstuffs (Kirschenbaum et al., 2000); their presence being due to the decarboxylation of amino acids by microorganisms (Rice and Koehler, 1976). Some of these compounds, such as methylamine, tyramine, and histamine, have previously been shown to occur in the ruminal fluid of sheep and cattle (Dain et al., 1955; van der Horst, 1961) as well as in the intestinal contents of pigs and humans. They have not previously been studied.

Figure 2. Effects of carbohydrate overload on amine concentrations in equine cecal contents incubated anaerobically in vitro. The concentrations of (a) phenylethylamine, (b) isoamylamine, (c) putrescine, and (d) cadaverine were measured by high performance liquid chromatography. Cecal contents were divided into aliquots and incubated for 24-h with the inclusion of either inulin (1 g/100 mL; ▼), cornstarch (1 g/100 mL; ▲), or without added carbohydrate (control; ■). Each value represents the mean ± SEM of estimates taken from four separate experiments. *Significant difference compared with control values, two-way repeated measures analysis of variance with Bonferroni’s post hoc test.
in the intestines of horses, although it has been speculated that their presence may produce physiological effects (Callingham and Williams, 1987).

Due to their structural similarities with endogenous amines, such as the catecholamines and 5-HT, a number of the amines found in equine cecal contents if released into the circulation could have effects on vascular function. The most important of these is likely to be peripheral vasconstriction; aromatic amines such as tyramine and phenylethylamine have been implicated as pressor agents in the pathogenesis of migraine in humans, following ingestion of foodstuffs such as cheese or wine (Shaw et al., 1978; Gonsalves and Johnson, 1977). In the horse, these compounds have been shown to cause the constriction of isolated digital arteries and veins, possibly via stimulation of 5-HT receptors or by displacement of norepinephrine from perivascular nerves, and, in addition, they may indirectly promote vasoconstriction by displacing serotonin from platelet stores (Bailey et al., 2001). Compounds or toxins that have the potential to cause peripheral vasoconstriction may be significant in the pathophysiology of laminitis because digital vasoconstriction has been proposed as the initiating event following fermentation in the cecum (Moore and Allen, 1996).

The present study showed that both cornstarch and inulin (a naturally occurring form of fructan carbohydrate of a type similar to that found in grass) were fermented by cecal bacteria, associated with a time-dependent drop in pH and a concomitant increase in production of a number of amines. Cornstarch, administered by stomach tube, has been used in many studies to induce acute laminitis in horses, which tends to occur within 36 to 48 h (Garner et al., 1975), and it has been suggested recently that fructans may be responsible for initiating pasture-induced laminitis (Longland et al., 1999), although small quantities may be beneficial to the balance of the hindgut flora (Rao, 1999).

### Table 1. Effects of virginiamycin and calcium hydrogen phosphate on the changes in pH and amine concentrations (micromolar) in equine cecal contents induced by incubation with starch or inulin over a 24-h period. Each value represents mean ± SEM of paired estimates taken from four separate experiments.

<table>
<thead>
<tr>
<th>Item</th>
<th>Negative control</th>
<th>Starch (positive control)</th>
<th>Starch + virginiamycin</th>
<th>Starch + CaHPO₄</th>
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<td>168.5 ± 10.0</td>
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<td>112.4 ± 23.9</td>
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<td>32.4 ± 5.7</td>
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<td>Cadaverine</td>
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<td>49.4 ± 19.8</td>
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<th>Inulin + CaHPO₄</th>
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*aSignificant difference compared with negative control value at 24 h.  
^b,c,d,e,f,g Significance difference compared to appropriate positive control at 24 h, one-way analysis of variance with Fisher's multiple comparison (P < 0.05).
The in vitro model used in the current study was similar to other studies examining rumen contents from cattle and sheep, in which an excess of fermentable carbohydrate will also cause toxicity and acidosis (Demeyer et al., 1996). Indeed, the bacterial flora of the equine cecum shows many similarities with that of the bovine rumen (Kern et al., 1974). Cecal contents were not diluted in these experiments; thus, the changes recorded in the current study may represent an underestimation of changes in vivo. Amine production may have been restricted to some extent due to limiting concentrations of amino acid substrates and to product inhibition.

The fairly stable pH and amine concentrations within control aliquots in the present study suggested that the conditions used did not have adverse effects on the major populations within the bacterial flora over the duration of the incubations. The addition of starch or fructans to the cecal contents resulted in a marked fall in pH over the time course of the experiments, the magnitude of which is consistent with the cecal pH changes demonstrated in vivo when horses have been administered large amounts of starch (Moore et al., 1979). In the present study, inulin caused a more rapid fall in pH than an equal amount of cornstarch, which may reflect differences in the ease of assimilation of these two substrates by the fermenting bacteria. Fructans have been shown to cause fermentation in the ceca of monogastric animals, such as the rat (Remesy et al., 1993), due to the fact that such oligosaccharides are not digestable in the mammalian small intestine. The variation of fructan levels in grasses under different climatic conditions is thought to account for the seasonal incidence of acute laminitis (Longland et al., 1999).

It was assumed that the pH changes described in the present study were primarily due to lactic acid production, although organic acids were not measured; lactic acidosis has been considered an important factor in the development of laminitis (Garner et al., 1977). It has been shown that cecal contents incubated with starch (pH 5.9) may produce an increase in the permeability of the equine hindgut to high molecular weight compounds (Weiss et al., 2000). This may facilitate the transfer of many potentially toxic compounds from the intestinal lumen into the circulation; however, it is not known to what extent the mucosal permeability to amine compounds may be affected under these conditions.

The production of several amines was increased, relative to controls, with the fermentation of starch or fructans. Most notably, phenylethylamine and isoamylamine concentrations increased in a linear fashion over the 24-h, by which time their concentrations were two- to threefold greater than the negative control values, which remained fairly constant. The concentrations of the diamines, putrescine and cadaverine, also increased in response to the addition of carbohydrate, but this effect was delayed and only apparent toward the end of the incubation.

Gram-positive bacteria such as Streptococci and Lactobacilli, as well as Gram-negative species, have the ability to produce amines from amino acids (Golovnya et al., 1969; Bover-cid and Holzapfel, 1999). In experimentally induced carbohydrate overload in the horse, it has been shown that cecal Gram-positive bacteria overgrow (Garner et al., 1978), suggesting that they are responsible for lactate production. It is likely that these bacteria, such as Streptococcus bovis and Lactobacilli sp. also contain the decarboxylase enzymes necessary to produce amines; however, it should be noted that the normal cecal concentrations of these amines are already in the micromolar range, so other bacterial species may have this capability. Further work is necessary to identify the bacterial species responsible for amine production in this model system.

The streptogrammin antibiotic virginiamycin partially inhibited the fall in pH associated with carbohydrate excess, most markedly with starch. It also inhibited the increase in production of phenylethylamine and isoamylamine seen with carbohydrate fermentation, and inhibited putrescine production (starch only), suggesting that production of the former two amines at least may be associated with Gram-positive bacteria. Virginiamycin was included at a concentration of 1 mg/100 mL, based upon the concentration shown to have effects on rumen fermentation in vitro (Marounek et al., 1995) and its MIC for Streptococcus bovis and Lactobacillus sp. cultured from the rumen (Nagaraja and Taylor, 1987). Orally administered virginiamycin moderates rumen fermentation in vivo (Coe et al., 1999), and in the horse this antibiotic has been marketed in Australia as Ponderguard, an oral formulation for the prevention of laminitis, which has been shown to suppress lactic acid production in the hindgut of horses caused by grain overload (Rowe et al., 1994).

As a comparison to virginiamycin, the effects of calcium hydrogen phosphate (CaHPO₄) and a source of plant steroidal saponins (extract of Yucca schidigera) were also investigated. Dietary calcium in the form of CaHPO₄ has been shown to modulate inulin-induced cecal fermentation in the rat, partly by control of luminal pH (Remesy et al., 1993). In the present study, CaHPO₄ significantly limited the fall in pH associated with fermentation of both starch and inulin by equine cecal contents. The small inhibition in amine production observed in the presence of CaHPO₄ did not reach statistical significance. Taken together, these results would tend to suggest that the significant effects of virginiamycin on amine production were due to specific antimicrobial properties, rather than being indirect through the moderation of pH.

Steroidal saponins, such as those extracted from the desert plant Yucca schidigera, have been shown to inhibit the growth of a number of Gram-positive bacteria, isolated from the cattle rumen, by altering their cell wall structure (Wang et al., 2000). One of the bacteria whose growth was partially inhibited was Streptococcus bovis, which has been found to overgrow in the hindgut of horses given carbohydrate overload and therefore implicated in the pathogenesis of laminitis (Garner et al.,
eral circulatory disturbances, which may be of relevance carbohydrate overload may be sufficient to cause peripheral absorption of such vasoactive compounds from the hindgut hydrogen phosphate or was inhibited by virginiamycin, but not by calcium hyphenylethylamine and isoamylamine. Their production increased production of a number of amines, most notably causes not only a fall in pH but also results in the inmentation of starch or fructans in the hindgut of horses in concentrations greater than 1\mu M. Furthermore, fermentation of starch or fructans in the hindgut of horses causes not only a fall in pH but also results in the increased production of a number of amines, most notably phenylethylamine and isoamylamine. Their production was inhibited by virginiamycin, but not by calcium hydrogen phosphate or Yeucca schidigera extract. The absorption of such vasoactive compounds from the hindgut into the circulation of the horse following ingestion of a carbohydrate overload may be sufficient to cause peripheral circulatory disturbances, which may be of relevance to the pathogenesis of laminitis.

### Implications

This study has demonstrated the presence of a variety of amine compounds in equine cecal contents, present in concentrations greater than 1\mu M. Furthermore, fermentation of starch or fructans in the hindgut of horses causes not only a fall in pH but also results in the increased production of a number of amines, most notably phenylethylamine and isoamylamine. Their production was inhibited by virginiamycin, but not by calcium hydrogen phosphate or Yucca schidigera extract. The absorption of such vasoactive compounds from the hindgut into the circulation of the horse following ingestion of a carbohydrate overload may be sufficient to cause peripheral circulatory disturbances, which may be of relevance to the pathogenesis of laminitis.

### Literature Cited


