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Dietary fructan carbohydrate increases amine production in the equine large intestine: Implications for pasture-associated laminitis[1]

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ABSTRACT: Pasture-induced laminitis in the horse is associated with the overconsumption of fermentable carbohydrate, in the form of simple sugars, fructans, or starch. The fermentation of carbohydrate in the cecum and large intestine results in the production of lactic acid and other toxins or "laminitis trigger factors." Vasoactive amines have been suggested as possible initiating factors. The aim of this study was to feed a commercially available form of fructan carbohydrate (inulin, 3 g/kg of BW per day) to normal ponies and to ponies predisposed to laminitis, to mimic a change from a basal hay diet to lush spring-summer pasture. Five normal and 6 laminitis-prone, native-breed ponies were acclimated to a basal hay diet before the inclusion of inulin and chopped dried grass. Blood samples, fecal samples, and foot temperature measurements were taken throughout the study. Amines were measured in the feces and plasma by HPLC and liquid chromatography-mass spectrometry, respectively. The pH of the fecal samples decreased from 6.89 ± 0.11 on the hay diet to a minimum of 6.18 ± 0.11 with the addition of inulin ($P < 0.05$). An increase was observed in the fecal concentrations of a number of amines, including tryptamine (2.5-fold increase, $P < 0.05$) and tyramine (2-fold increase, $P < 0.05$). No changes were noted in plasma amine concentrations or plasma D- or L-lactate, indicating that there may be a threshold of hindgut pH change before mucosal damage can result in the release of these factors into the circulation. No differences in pH or any of the measured compounds were observed between the group of normal ponies and those predisposed to laminitis. This indicates that differences in the intestinal microflora do not account for this predisposition. However, the results from this study indicate that moderate increases in dietary fructan carbohydrate can produce increases in bacterial fermentation products and other compounds in the large intestine, which may be relevant to the pathogenesis of acute laminitis in ponies on pasture.

Key words: amines, fructans, laminitis, pony

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INTRODUCTION

Laminitis is one of the most common conditions affecting the equine population, and it can render animals permanently lame, sometimes necessitating euthanasia. In groups of ponies kept on the same pasture, certain individuals may be predisposed to recurrent acute episodes of laminitis, whereas others are rarely or never affected (Treiber et al., 2006).

Most laminitis cases are associated with gastrointestinal disturbances, and causes include the overconsumption of fermentable carbohydrates such as cereal grains or lush pasture (Hood et al., 1993). One important form of carbohydrate in pasture that may be involved in the onset of laminitis is fructans (Longland and Byrd, 2006). Large amounts of fructo-oligosaccharides (lower molecular weight fructans) given by gavage have been shown experimentally to induce acute laminitis (van Eps and Pollitt, 2006). Fructan fermentation, primarily in the cecum and large intestine, results in the production of lactic acid and other toxins or "laminitis trigger factors," which enter the circulation and cause changes to the lamellar tissues that suspend the distal phalanx from the hoof wall (Elliott and Bailey, 2006). Among the potential trigger factors formed in the hindgut are amine compounds. These are formed by the decarboxylation of amino acids by many of the same gram-positive bacteria that ferment carbohy-

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drates. In the circulation, these compounds mimic endogenous amines, such as serotonin and norepinephrine, and can cause peripheral vasoconstriction (Elliott et al., 2003; Bailey et al., 2004).

The aim of this study was to feed inulin, a commercially available form of fructan carbohydrate, to normal ponies and to ponies predisposed to laminitis to mimic a change from a basal hay diet to lush spring-summer pasture. The objective was to monitor changes in fecal pH and to determine whether fecal or plasma concentrations of amines, or both, increased. Furthermore, differences between normal and laminitis-prone ponies were investigated. It should be noted that the amounts of fructan fed to these animals was well below that required experimentally to induce laminitis (van Eps and Pollitt, 2006), and it was not the intention of this study to reproduce the disease.

MATERIALS AND METHODS

Animals

Studies were conducted under a United Kingdom Home Office license and with the approval of the Ethics and Welfare Committee of the Royal Veterinary College (London, UK).

The animals used for this study consisted of mixed, adult native-breed ponies. The mean BW was 337 ± 36 kg, and the mean age was 13.1 ± 0.9 yr. Each animal was assessed for overall BCS using a previously validated scoring system (Henneke et al., 1983), and those with similar scores were selected for the study (i.e., excluding any with a score of ≤4 or ≥7). The animals were divided into 2 groups: normal (n = 6) and laminitis-prone (n = 6). The normal ponies were defined as those with no history or radiographic signs of previous episodes of laminitis. Ponies in the laminitis-prone group were selected on the basis of having had acute episodes of pasture-induced laminitis at least once in the previous 3 yr, but with no clinical signs in the 3 mo leading up to the study.

The study was designed such that each pony served as its own control. Ponies were managed in pairs, consisting of 1 normal and 1 laminitis-prone pony matched for age, weight, and sex. At the beginning of the study, 1 of the normal ponies had to be euthanized for reasons unrelated to this project; therefore, 1 laminitis-prone pony was not matched.

Diets

The duration of the trial was 3 wk in total for each cohort. The control (lower carbohydrate) diet was fed in wk 1 and 2. The animals were taken from pasture, housed in separate stalls, and fed hay and water ad libitum; the amount consumed by each animal was recorded daily. Each pair was fed from the same bale of hay, which had previously been analyzed for water-soluble carbohydrate content. At the beginning of wk 3 (d 0), the animals were switched to a greater carbohydrate diet, the composition of which was formulated to emulate spring grass. Three grams per kilogram of BW of inulin (a dietary fructan, originated from chicory root; degree of polymerization ranged from 2 to 60) was included to increase dietary fructan consumption. Inulin was purchased from Orafti Group (Tienen, Belgium). Pilot studies using 0.3 and 1 g of inulin/kg of BW (single feed) were conducted first to estimate the final dose of 3 g/kg of BW.

The forage ration consisted of two-thirds of the daily forage intake by weight (calculated for each individual animal from wk 1 and 2) as hay, plus one-third chopped, dried grass. The dried grass (*Lolium perenne*; Readigrass) obtained from Spillers Effem Equine Ltd., Milton Keynes, UK) was added as a protein source (contained 15% protein), such that amino acid concentrations would not be a limiting factor in amine production. The inulin and dried grass components of the diet for each day were combined and divided into 3 meals given at 0800, 1300, and 1600. The inulin was in powder form, and it was added to the dried grass, which had been wetted with water in a feed bucket. The grass plus inulin was completely consumed and appeared to be palatable. The hay was eaten intermittently during the day and night.

Sampling

Samples were taken at 6, 5, 4, and 2 d before the inclusion of inulin in the diet, and 1, 2, 3, and 5 d after the diet change (designated as time 0). On each of these days, blood and fecal samples were taken simultaneously at 1700; hoof wall and coronary band temperatures were also recorded, and the animals were assessed for any clinical signs that might indicate laminitis.

Hoof Wall and Coronary Band Temperature Measurement

The surface temperature, measured at the hoof wall or coronary band, has previously been validated as a noninvasive index of digital blood flow (Bailey et al., 2004) and was used to ensure that hemodynamic disturbances that might lead to acute laminitis were avoided. Hoof wall and coronary band temperatures of each animal were recorded on each sampling day. The animals were taken to a temperature-controlled room (20 ± 1°C). After a 1-h acclimation period, an infrared thermometer (Model IT-304, Horiba ABX, Montpellier, France) was used to record the temperature from a marked point on the hoof and a clipped area 1 cm dorsal on the coronary band. Recordings were taken from each limb every 15 min until the temperature stabilized as indicated by 3 consecutive measurements within a 0.5°C range. During this time, each animal was examined for sole tenderness and digital pulse to ensure that there were no clinical signs of hoof sensitivity, which would indicate laminitis.

Fecal pH

Fecal samples were collected to measure fecal pH and fecal amine concentrations. Approximately 20 g of freshly passed feces was collected from each animal into sterile tubes. Two grams of sample was placed into a small plastic bottle and the same weight of deionized water was added. The contents of the tube were mixed thoroughly by vortexing for 20 s, and the pH was measured using a pH meter. The remainder of the fecal sample was stored at –80°C for amine analysis.

Fecal Amine Measurement

The concentrations of amines in the feces were measured by adapting an HPLC method for detecting cecal amines that was used by Bailey et al. (2003b). Once thawed, 5 g of sample was added to 8 mL of water. The contents were mixed thoroughly and centrifuged at 2,000 × *g* for 15 min at 4°C. Approximately 5 mL of supernatant was decanted and recentrifuged at 2,000 × *g* for 15 min at 4°C. The clear supernatant (2.5 mL) was adjusted to 5 mL with acetone:water (2:1, vol/vol). The internal standard (heptylamine, 5 μg/mL) was added to each sample before the addition of 1 mL of borax buffer (3.81 g of sodium tetraborate in 100 mL of water adjusted to pH 10.5 with 10 *M* sodium hydroxide) to make the solution basic. Amines were derivatized by adding 2 mL of dansyl chloride (1%, wt/vol, in acetone) and the overall volume was adjusted to 10 mL with acetone:water (2:1, vol/vol). The solution was shaken and placed in a water bath at 65°C for 25 min.

Derivatized amines were extracted using a solid-phase extraction process, as previously described (Sep-Pak C18 cartridges, Waters Ltd., Elstree, UK; Bailey et al., 2003b). Samples were eluted with 2 mL of acetonitrile. Chromatography was carried out using a liquid chromatograph system that comprised a gradient controller and pump with a UV absorbance detector (model 600E and model 484, respectively, Waters Ltd.). Separation of the compounds was carried out using a reverse-phase C18 column (Symmetry Shield, 150 × 3.9-mm i.d., particle size 5 μm; Waters Ltd.). The gradient consisted of 25% acetonitrile, increasing to 70% over a 50-min run with a flow rate of 0.7 mL/min. All solvents were of HPLC analytical grade. The compounds were detected by measuring UV absorbance at 250 nm. The amines from the fecal samples were identified and quantified according to their retention times and compared with standard curves constructed from pure amines (free base form, 0 to 100 μg/mL; Sigma Chemicals, Poole, Dorset, UK). The concentrations of the derivatized amines were calculated from the area under the curve for the amine divided by the area under the curve for the internal standard. The fecal samples were dried and weighed, and the amine concentration was determined by HPLC in each sample was calculated as micrograms per gram of DM.

Plasma Amine Measurement

Blood samples were also taken at the same time to measure plasma amine and lactic acid concentrations. Approximately 20 mL of blood was collected from the jugular vein of each pony into heparinized tubes containing 50 μL of 0.1 m*M* clomipramine and 50 μL of 1 m*M* phenelzine sulfate to inhibit platelet amine uptake and metabolism, respectively. The blood was centrifuged at 300 × *g* for 10 min at 4°C to give platelet-rich plasma. The platelet-rich plasma was separated and centrifuged at 10,000 × *g* for 10 min to give platelet-poor plasma, which was then stored in aliquots at –80°C for future amine analysis. It was necessary to separate platelets so that these did not rupture on freezing and release amines into the plasma.

The concentrations of tryptamine, phenylethylamine, isoamylamine, and tyramine were measured in plasma samples by liquid chromatography combined with mass spectrometry, as previously described (Bailey et al., 2003c). Pure amine standard solutions were prepared in the range of 0 to 500 ng/mL. Heptylamine was added to 1 mL of plasma or 1 mL of standard to give an internal standard concentration of 50 ng/mL. All samples were adjusted to 5 mL using acetone:water (2:1, vol/vol). The plasma samples were centrifuged at 2,000 × *g* for 10 min at 4°C to remove the precipitated proteins, and the supernatant was collected. All samples were prepared and the amines derivatized using the method described for the fecal samples. The amines were extracted using the solid-phase extraction protocol as previously described, and the eluted samples were evaporated to dryness under a stream of nitrogen at 70°C and reconstituted in 100 μL of 50:50 (vol/vol) acetonitrile:0.1% formic acid in HPLC-grade water. Chromatography was carried out using a reverse-phase C18 column as described for the fecal amines. The mobile phase initially consisted of 25% acetonitrile, 74.9% water, and 0.1% formic acid and changed to 70% acetonitrile, 29.9% water, and 0.1% formic acid at 5 min. Atmospheric pressure, chemical ionization, mass spectrometry (Micromass Platform LC API mass spectrometer system, Micromass Ltd., Manchester, UK) was used to identify and quantify the amines according to their retention time and molecular weight. The amine concentrations in the samples were calculated relative to the internal standard using the calibration curve.

Lactic Acid Measurement

The lactic acid concentrations in the plasma were measured using an enzymatic colorimetric assay (DL-lactic acid kit, Biosentec, Toulouse, France), according to the manufacturer's instructions. Wavelengths of the samples were measured using a plate reader set at 340 nm.

Statistical Analysis

Plasma amine concentration and standard curve measurement were calculated using Masslynx software

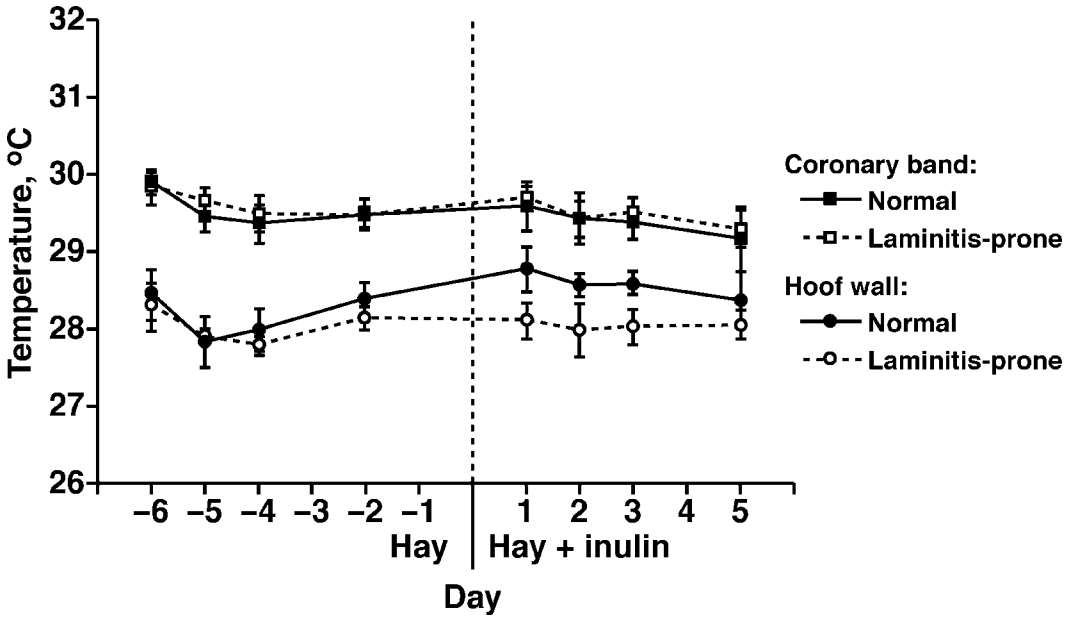

Figure 1. Changes in hoof wall (circles) and coronary band (squares) temperatures of normal ponies and ponies predisposed to laminitis on a basal hay diet and after the inclusion of inulin (3 g/kg of BW per day). Each point represents the mean ± SEM of 5 normal ponies (solid symbols) or 6 laminitis-prone ponies (open symbols).

(version 3.0 for Windows, Micromass Ltd.). All other curve fitting and statistical comparisons were performed using GraphPad Prism (version 3.0 for Windows, GraphPad Software, San Diego, CA).

All data were expressed as means ± SEM. The difference in hoof wall and coronary band temperatures, fecal pH, and fecal amines on the inulin diet compared with the hay diet was calculated using a 1-way, repeated-measures ANOVA followed by a Dunnett's post hoc test to compare values from samples taken on the first sampling day (d –6) on the hay diet to values obtained on all other days. The difference in plasma amine and lactic acid concentrations between the hay and inulin diets was calculated using a paired *t*-test. Differences between the data from normal and laminitis-prone ponies were assessed using 2-way ANOVA followed by Bonferroni's post hoc test. Statistical significance was accepted at $P < 0.05$. Statistical power was determined using online software (UCLA Department of Statistics, 2006).

RESULTS

Assessment of Ponies and Diet

The average BCS were 5.9 for normal ponies and 5.8 for those predisposed to laminitis, categorizing all ponies into the moderate range. Each individual pony displayed a good appetite throughout the study, and all feeds offered were consumed. Calculations of total fructan consumption while on the basal hay diet resulted in a mean value of 4.30 ± 0.26 g/kg of BW per d, which increased to 6.15 ± 0.23 g/kg of BW per d with the inclusion of inulin. No animal on this study showed any signs of lameness or laminitis, although a transient increase in the digital pulse pressures in 1 or 2 digits was noted in some ponies.

Hoof Wall and Coronary Band Temperatures

The hoof wall and coronary band temperatures recorded throughout the trial are presented in Figure 1. Each point represents mean ± SEM from 5 normal ponies and 6 ponies predisposed to laminitis. There was no difference in coronary band or hoof wall temperatures recorded on the hay diet compared with the inulin diet in either group. Furthermore, there was no difference in the hoof wall or coronary band temperatures of normal compared with laminitis-prone ponies at any time.

Fecal pH

The pH of the fecal samples decreased when animals switched from the control hay diet to the hay diet with added inulin (3 g/kg per day; Figure 2). The pH fell from 6.89 ± 0.11 on the hay diet (d –4) to a minimum of 6.18 ± 0.11, which was reached after 2 d of inulin feeding ($P < 0.001$). Pilot studies including inulin at 1 g/kg per day also produced a decrease in pH, declining from 6.97 ± 0.10 to a minimum of 6.29 ± 0.11 ($P < 0.05$).

When the normal and laminitis-prone ponies were considered separately, no differences were observed between the 2 groups in fecal pH over the course of the trial (inset, Figure 2). The fecal pH of both groups increased again slightly at d 3 and 5.

Fecal Amines

Fourteen amines were identified and quantified in the fecal samples from all animals. They consisted of

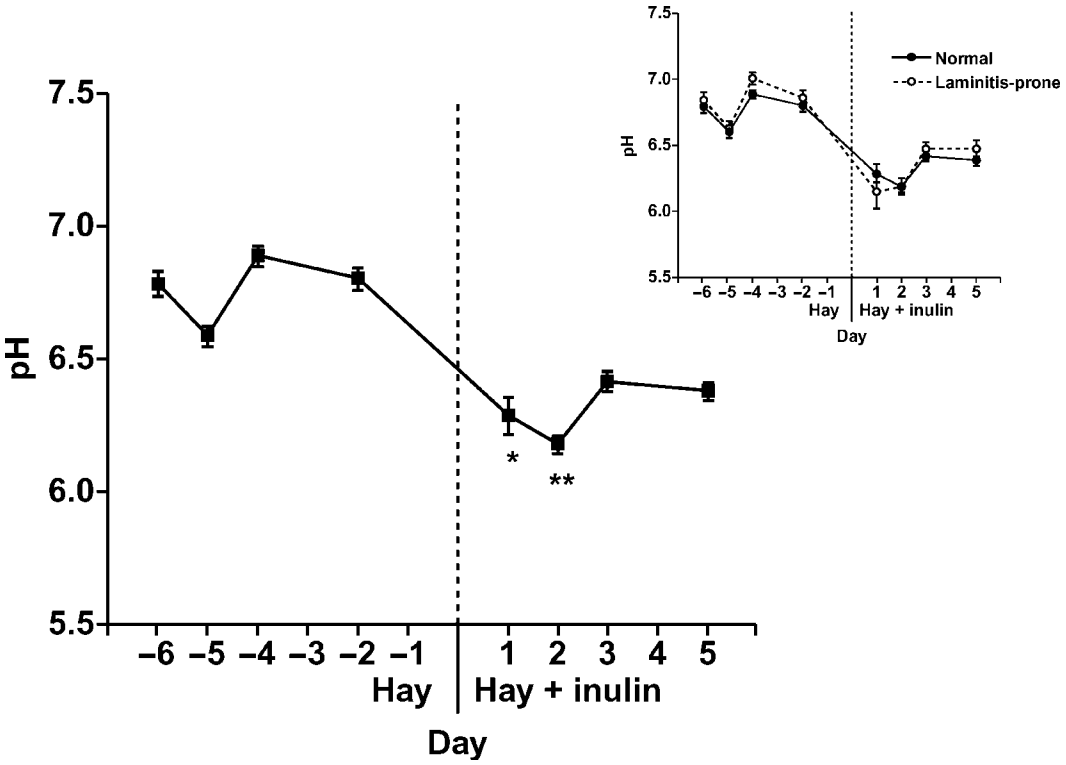

Figure 2. The effect of dietary inulin (3 g/kg of BW per day) on fecal pH of normal and laminitis-prone ponies. In the main figure, each point represents the mean ± SEM of 11 animals; in the inset, each point represents the mean ± SEM of 5 normal ponies (solid symbols and line) or 6 laminitis-prone ponies (open symbols, dashed line). Difference compared with the value on the first sampling day (d –6) was based on 1-way ANOVA with Dunnett's post hoc test; *$P < 0.05$ and **$P < 0.001$.

the aliphatic monoamines: methylamine, ethylamine, propylamine, isoamylamine, and isobutylamine; the aromatic monoamines: tryptamine, tyramine, and phenylethylamine; and the diamines: putrescine, cadaverine, histamine, diaminoheptane, spermidine, and spermine (Bailey et al., 2003b). There were no differences in the concentrations of methylamine, ethylamine, propylamine, cadaverine, histamine, spermidine, and spermine found following the inclusion of inulin in the diet (data not shown). An increase was observed in the concentrations of tryptamine (2.5-fold increase between d –4 and d 2), and tyramine (2-fold increase; $P < 0.05$; Figure 3). For these 2 amines, the increase was evident after 24 h on the inulin diet. Phenylethylamine and isobutylamine were also increased on the inulin diet compared with the hay diet (Figure 3), as were putrescine and diaminoheptane (data not shown).

Sustained differences between the normal and laminitis-prone ponies were observed only for isobutylamine; however, this difference was observed before the introduction of inulin (inset, Figure 3). Tryptamine concentrations between the 2 groups were similar except for those observed on d 2 (inulin diet), when the concentrations were lower in the laminitis-prone group.

Plasma Amines

The 4 plasma amines measured (phenylethylamine, tyramine, tryptamine, and isoamylamine) were quantifiable in the plasma samples from all of the ponies in the study. Their concentrations are shown in Figure 4. There was no difference in the concentrations of any of the amines in the plasma of ponies on the hay diet (d –4) compared with the inulin diet (d 2). Furthermore, there were no differences in the plasma amine concentrations between the normal and laminitis-prone groups (inset, Figure 4).

Plasma Lactate

Both D- and L-lactic acid could be detected in plasma samples, but at low concentrations. No increase in D-lactate was observed in either group of ponies following the inclusion of inulin in the diet at 3 g/kg per day (Figure 5), indicating that the integrity of the intestinal mucosa remained intact; indeed, a decrease in D-lactate was recorded on d 2 compared with d –4 ($P < 0.05$). There was no difference in the L-lactic acid concentrations of ponies fed the hay diet compared with the inulin diet.

DISCUSSION

The results from this in vivo dietary study demonstrated that it was possible to detect changes in quantities of various metabolites present in the large intestine in response to feeding fructan as inulin at 3 g/kg of BW per day. Some of these changes may be relevant to the

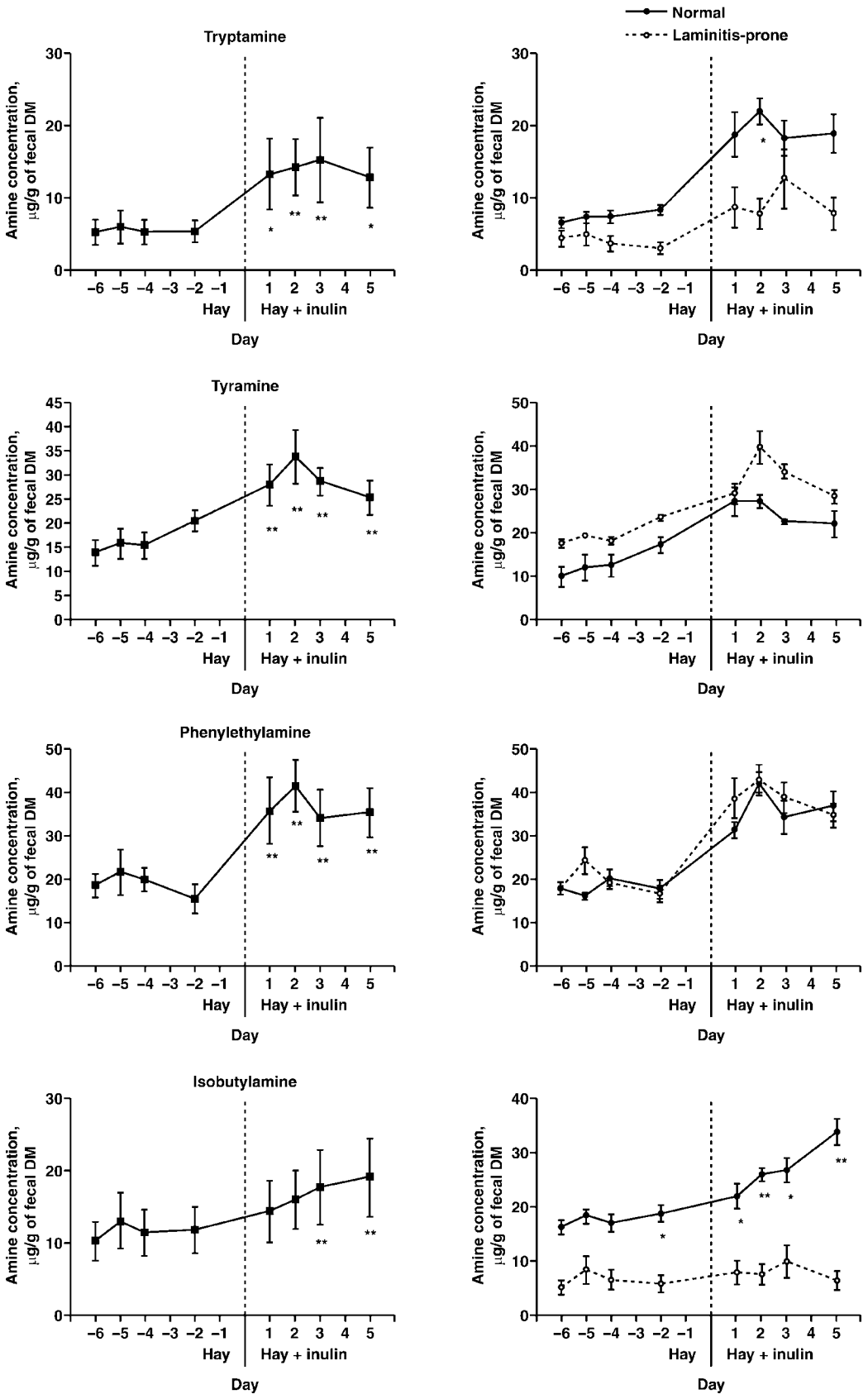

Figure 3. Amine concentrations in fecal samples from ponies fed hay diets and after supplementation with inulin (3 g/kg of BW per day). In the main figure, each point represents the mean ± SEM of 11 animals; in the inset, each point represents the mean ± SEM of 5 normal ponies (solid symbols and line) or 6 laminitis-prone ponies (open symbols, dashed line). Difference compared with the value on the first sampling day (d –6) was based on 1-way ANOVA with Dunnett's post hoc test; *$P < 0.05$ and **$P < 0.001$.

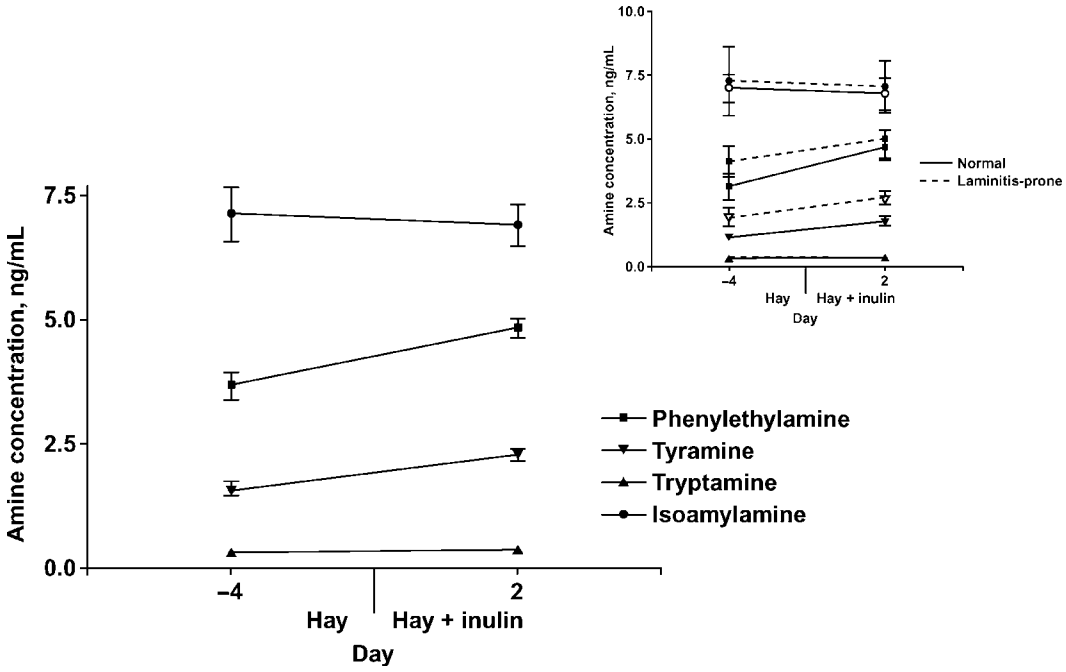

Figure 4. The concentrations of phenyethylamine (■), tyramine (▲), tryptamine (▼), and isoamylamine (●) in plasma from ponies sampled on d –4 (hay diet) and d 2 (inulin diet). Each point represents the mean ± SEM of 11 individuals. The inset shows plasma amine concentrations for normal (—; n = 5) and laminitis-prone (—; n = 6) ponies.

pathogenesis of acute laminitis in ponies at pasture. Dose-dependent changes in fecal pH, together with the formation of vasoactive amines, were observed. These changes, however, were not reflected in alterations in plasma lactate or amines, or in any clinical signs. This indicates that the threshold for inulin inclusion, at which point hindgut alterations lead to significant leakage of these factors into the circulation, is somewhat above 3 g/kg of BW. However, such an intake of fructan might be attainable by ponies on lush pasture (Longland and Byrd, 2006).

It should be noted that inulin, the fructan used in the current study, might have some structural differences compared with levan-type fructans found in grasses. Both are β-linked D-fructofuranosyl fructans; however, the inulins are generally of a lower molecular weight (3,000 to 5,000 Da) and are composed of β-D-fructofuranosyl units with (2-1)-linkage, whereas levans (16,600 to 33,200 Da) are composed of βD-fructofuranosyl units with (2-6)-linkage (Vijn and Smeekens, 1999). In the current study, this commercially available material was used to represent starch, sugars, and fructans that reach the hindgut and can be fermented rapidly to produce lactic acid. It is, therefore, a model of the possible pathogenic pathway rather than an exact representation of pasture-induced laminitis. There may be some differences in prececal degradation and microbial fermentation of these 2 fructan types. In vitro digestibility models have shown that grass fructans may be partially susceptible to acid hydrolysis in the same way as inulin (J. Ince and A. Longland, Univ. Wales, Aberystwyth, UK; personal communication); however, grass fructans have not yet been extracted in large enough quantities to enable them to be used in whole-animal studies.

It has previously been shown that addition of inulin to equine cecal contents, which were incubated anaerobically in vitro, caused a decrease in pH and an increase in amine production over 24 h (Bailey et al., 2002). The pH in the feces and terminal colon has been shown to be a reasonable indicator of the pH in the cecum and proximal colon (Kern et al., 1974; Berg et al., 2005). Therefore, it can be inferred that the pH of the cecum and large colon of these ponies also decreased, because of fermentation of the increased concentrations of fructans in the test diet. However, some diurnal fluctuation in fecal pH was noted in the current study, and, therefore, it is likely that there may have been some variation in fecal sample composition, including amine concentrations at different times of the day. The pH change is thought to be due primarily to the production of lactic acid from the fermentation of nonstructural carbohydrates by gram-positive bacteria, as has been documented following excess starch administration in vivo (Garner et al., 1977, 1978). The lactic acid-producing bacteria that ferment carbohydrates in the horse gastrointestinal tract have recently been identified by molecular means and include *Lactobacillus* spp. and *Streptococcus* spp. (Al Jassim et al., 2005; Milinovich et al., 2006).

The fecal pH in the current study decreased by nearly 0.75 of a log unit 24 h after the beginning of feeding the test diet. This was appreciably smaller than the pH change observed with laminitis experimentally induced by fructan overload, in which 7.5 to 12.5 g/kg of BW of

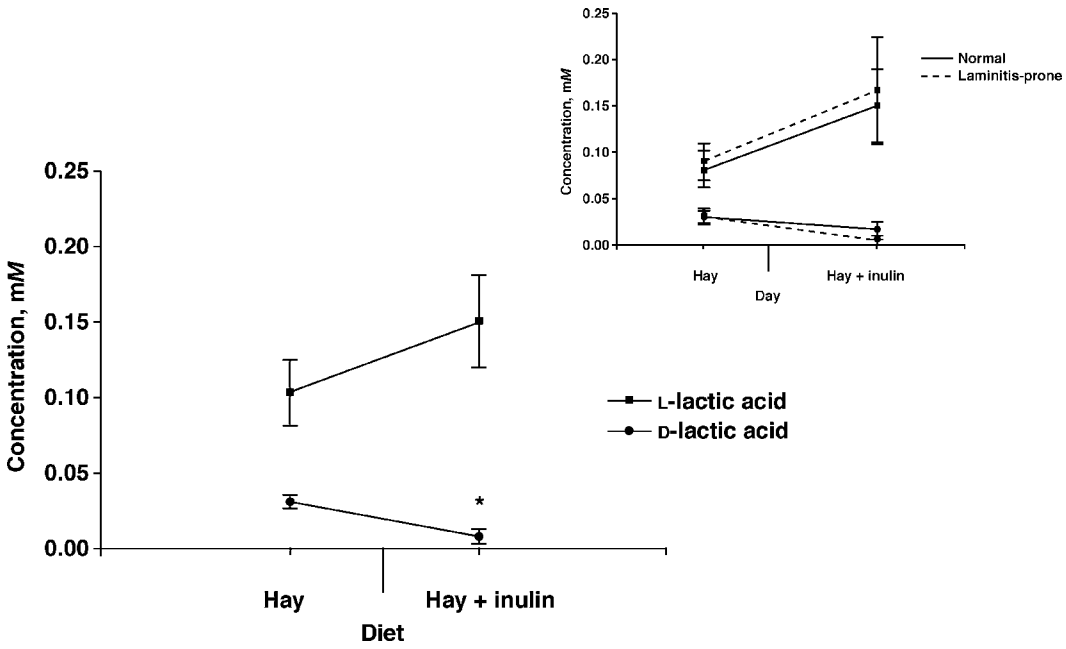

Figure 5. Concentration of D- (●) and L-lactic acids (■) in plasma from ponies fed hay diets (day –4) and after supplementation with inulin (3 g/kg of BW per d; day +2). Each point represents the mean ± SEM of 11 individuals. The inset shows lactic acid concentrations for normal (—; n = 5) and laminitis-prone ponies (—; n = 6). *Difference compared with the hay diet (paired *t*-test, $P < 0.05$).

oligofructose (raftilose; degree of polymerization 2 to 8) was administered by gavage (van Eps and Pollitt, 2006), resulting in a decrease in fecal pH of 3 log units a short time before the onset of clinical signs.

The production of several amine compounds was increased in response to the increased dietary fructan carbohydrate in the current study. Tyramine, tryptamine, and phenylethylamine have been shown to have effects on equine digital blood vessels in vitro, causing contraction mediated by α-adrenoceptors or 5-hydroxytryptamine (serotonin) receptors or both (Elliott et al., 2003). Tryptamine and phenylethylamine have also been shown to reduce digital blood flow in Thoroughbred horses (Bailey et al., 2004). Others, such as isoamylamine and isobutylamine, may displace 5-hydroxytryptamine from platelets or prevent its clearance by the vascular endothelium (Bailey et al., 2003d; Elliott et al., 2003). Therefore, these compounds have been proposed as possible trigger factors linking gastrointestinal disturbances with the development of laminitis (Elliott and Bailey, 2006).

Amines are produced by the decarboxylation of amino acids by bacteria in the cecum and large intestine, and many of the same bacterial species that produce lactate and proliferate in the presence of rapidly fermentable carbohydrates are responsible for monoamine production (Bailey et al., 2003a). The current study considered whether any differences existed between the bacterial populations present in the intestinal tracts of normal ponies compared with those suffering periodic episodes of laminitis. Such differences might be reflected in the magnitude of changes in fecal pH or amine concentration when fed an excess of nondigestible carbohydrate. In terms of the fecal pH change due to lactate production from carbohydrate fermentation, no evidence was observed for differences between the 2 groups. However, this does not necessarily exclude the possibility of differences in the prececal fermentation of inulin. It has been recently shown that a significant amount of inulin may be fermented in the stomach and small intestine (Mößeler et al., 2005).

Similarly, no physiologically significant differences were detected between the groups of ponies with respect to changes in amine concentrations within the large intestines. These findings led us to the conclusion that differences among individuals, which render them more or less predisposed to recurrent episodes of laminitis due to fructan fermentation, may involve other factors. Such factors may include the barrier function of the intestinal mucosa that prevents toxins passing into the circulation, the detoxifying capacity of enzymes in the liver and the circulation (such as monoamine oxidases; Callingham and Williams, 1987), and the sensitivity of the lamellar tissues and blood vessels to the effects of laminitis trigger factors (Elliott and Bailey, 2006).

To investigate this further, the plasma concentrations of 4 amines (tryptamine, tyramine, phenylethylamine, and isoamylamine) were measured to determine the extent to which the increased production of these compounds in the intestinal contents was reflected in the plasma. A method for measuring these 4 amines in pony plasma had previously been validated, and plasma concentrations have been found to increase

during seasons of the year that are associated with high pasture fructan content (Bailey et al., 2003c).

Although the plasma concentrations of phenylethylamine and tryptamine showed an increasing trend following the inclusion of inulin in the diet at 3 g/kg of BW, this apparent increase was not statistically significant. The statistical power of the study was not sufficient to detect such a change with confidence; group sizes of 57 animals or more would have been required to do so. Access of these amine compounds into the circulation may be associated with altered mucosal barrier function brought about by decreasing pH; however, a threshold pH may have to be reached before significant transfer occurs. Following carbohydrate overload, acid damage to the cecal mucosa has been documented (Krueger et al., 1986), and Weiss et al. (2000) showed that the mucosal permeability of the colon becomes altered in vitro when incubated with cecal contents, in which the pH was lowered by lactate production due to the addition of cornstarch.

The D-isomer of lactic acid, produced principally by bacteria, may be measured in the plasma as a marker of intestinal damage or increased mucosal permeability following carbohydrate overload. In the developmental stages of oligofructose-induced laminitis (using bolus doses of 7.5 to 12.5 g/kg by stomach tube), significant quantities of Dlactate (2 to 3 mmol/L) have been observed in the plasma between 4 and 32 h after induction (van Eps and Pollitt, 2006). In the current study, however, there was no increase in plasma D-lactate concentration; in fact, there was a small decrease, with values maintained below 0.05 mmol/L. In addition, plasma L-lactate (which may also be produced by some bacterial species, such as *Streptococcus bovis*) was not elevated in the current study, and did not exceed 0.2 mmol/L.

These data indicate that the 0.75 log unit decrease in pH in the large intestine caused by the addition of 3 g of inulin/kg of BW to the ponies' diet was not sufficient to reach the threshold necessary for significant mucosal damage and increased permeability to occur. This is consistent with the observation that no increase in plasma amines was observed. In addition, there were no differences between the normal and laminitis-prone groups of ponies in any of the metabolites measured in plasma.

In light of the lack of changes in plasma concentrations of vasoactive amines, it was not surprising that no changes were observed in hoof wall or coronary band temperature throughout the study. The group of ponies predisposed to laminitis used in the current study did tend to exhibit a slightly lower hoof wall temperature than the normal group, although this was not statistically significant. Further work would be required to determine if this finding is a true difference. Decreased digital blood flow in these individuals or by vascular damage and lamellar fibrosis caused by previous episodes of laminitis might account for these changes.

No signs of lameness attributable to laminitis were observed in this study. Presumably, doses of fructans much closer to 7.5 g/kg in one bolus are required before signs of laminitis would be observed, and the supplementary dose of 3 g/kg split into 3 meals was safely below this threshold. When relating this study to the situation of ponies at pasture, the question arises of how much fructan carbohydrate ponies might consume over a short period. It has been calculated from some studies of pasture consumption (Longland and Byrd, 2006) that horses can commonly consume between 2.1 and 3.5 kg of fructans per d (4.2 to 7.0 g/kg of BW) for a 500-kg animal. This calculation was based on DM intakes of between 1.5 and 3% of BW per d, and a peak fructan content of around 25 to 30% of DM, although greater DM intakes have been reported (Longland and Byrd, 2006). Therefore, even at the lower end of this range of DM intakes, the total intake of fructan at certain times of the year on certain pastures could be considerable. This is in addition to the other hydrolyzable carbohydrates (starch and simple sugars) in the pasture, which can also promote rapid hindgut fermentation (Potter et al., 1992).

Although large intakes of fructans may cause severe deleterious effects on equine health, small quantities might be beneficial. Feeding very small quantities of short-chain fructo-oligosaccharide (2 to 4 residues long; 8 or 24 g total/d, equating to approximately 0.02 or 0.06 g/kg of BW, respectively) to Quarter Horse yearlings has been shown to cause only a slight (0.1 log unit) drop in pH. This change was associated with increased volatile fatty acid concentrations and decreased numbers of *Escherichia coli* in the feces (Berg et al., 2005).

Factors such as insulin resistance may predispose individual animals to laminitis (Treiber et al., 2006), such that the threshold limit of fermentable carbohydrate consumption that causes laminitis may be reduced. Preliminary evidence from our group indicates that ponies in this study that were predisposed to periodic episodes of laminitis may exhibit elevated plasma insulin concentrations in response to dietary fructan, even with the inclusion of just 0.3 g/kg of BW (Bailey and Harris, 2006). Further work would need to be carried out using such animals to determine the amount of dietary fructan necessary to induce laminitis, compared with control animals, but this was outside the scope of the current study. Such understanding of predisposing factors may be beneficial in the design of effective countermeasures to prevent laminitis in affected animals (Harris et al., 2006).

In summary, the addition of fructan carbohydrate in the diet of UK native-breed ponies resulted in pH changes in the feces and increased bacterial production of amine compounds. Some of these amines are known to have vasoconstricting effects in the equine digit; however, the changes in the intestinal contents induced by these diets did not seem to cause damage to the intestinal mucosa or the release of any laminitis trigger factors. No differences in pH or any of the measured compounds were observed between the group of normal ponies and those predisposed to laminitis, indicating

that differences in the intestinal microflora or mucosal barrier do not account for this predisposition. This study increases our understanding of the changes in equine hindgut function caused by dietary fructans and may be useful as a model for investigating methods by which the bacterial production of amines and other potential laminitis trigger factors may be prevented.

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