

ORIGINAL ARTICLE

Microbial ecology of the equine hindgut during oligofructose-induced laminitis

Gabriel J Milinovich¹, Paul C Burrell², Christopher C Pollitt¹, Athol V Klieve³, Linda L Blackall⁴, Diane Ouwerkerk³, Erika Woodland¹ and Darren J Trott¹

¹Australian Equine Laminitis Research Unit, School of Veterinary Science, The University of Queensland, Brisbane, Queensland, Australia; ²Biosecurity Sciences Laboratory, Animal Research Institute, Biosecurity Queensland, Brisbane, Queensland, Australia; ³Department of Primary Industries and Fisheries, Animal Research Institute, Brisbane, Queensland, Australia and ⁴Advanced Water Management Centre, The University of Queensland, Brisbane, Queensland, Australia

Alimentary carbohydrate overload is a significant cause of laminitis in horses and is correlated with drastic shifts in the composition of hindgut microbiota. Equine hindgut streptococcal species (EHSS), predominantly *Streptococcus lutetiensis*, have been shown to be the most common microorganisms culturable from the equine caecum prior to the onset of laminitis. However, the inherent biases of culture-based methods are estimated to preclude up to 70% of the normal caecal microbiota. The objective of this study was to evaluate bacterial population shifts occurring in the equine caecum throughout the course of oligofructose-induced laminitis using several culture-independent techniques and to correlate these with caecal lactate, volatile fatty acid and degrees of polymerization 3–7 fructo-oligosaccharide concentrations. Our data conclusively show that of the total microbiota present in the equine hindgut, the EHSS *S. lutetiensis* is the predominant microorganism that proliferates prior to the onset of laminitis, utilizing oligofructose to produce large quantities of lactate. Population shifts in lactobacilli and *Escherichia coli* subpopulations occur secondarily to the EHSS population shifts, thus confirming that lactobacilli and coliforms have no role in laminitis. A large, curved, Gram-negative rod previously observed during the early phases of laminitis induction was most closely related to the *Anaerovibrio* genus and most likely represents a new, yet to be cultured, genus and species. Correlation of fluorescence *in situ* hybridization and quantitative real-time PCR results provide evidence supporting the hypothesis that laminitis is associated with the death en masse and rapid cell lysis of EHSS. If EHSS are lysed, liberated cellular components may initiate laminitis.

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Introduction

Laminitis constitutes the most serious condition of the equine foot, representing between 7.5% and 15.7% of all lameness problems (USDA, 2000). In the horse, laminitis results in basement membrane damage and eventual lamellar separation at the dermal–epidermal junction (French and Pollitt, 2004). This lamellar instability results in detachment of the distal phalanx from the inner hoof wall and, in severe cases, downward displacement and rotation of the bone. Under the animal's weight this damages the vasculature of the hoof, crushes the corium of the

sole and coronet, often necessitating euthanasia (Pollitt, 1999). The specific mechanisms by which lamellar separation occurs remain to be elucidated; however, the factors responsible for initiating laminitis are well described, the most common being carbohydrate overload through exposure to certain pastures or excess grain (USDA, 2000).

Consumption of pasture has been reported to account for in excess of 45% of laminitis cases (USDA, 2000). In some plants, most notably temperate grasses, fructans (β -D-fructose polymers with terminal glucose monomers) constitute the predominant form of storage carbohydrate (Roberfroid and Delzenne, 1998). Under certain climatic conditions, starch may account for as little as 10–13% of the total stored carbohydrate in these species, the remainder being sucrose and fructans (Cairns and Longland, 1998). Mammals are unable to metabolize fructans (Nilsson *et al.*, 1988) and in the equid, the fructans in grasses are rapidly transported through

Correspondence: GJ Milinovich, Australian Equine Laminitis Research Unit, School of Veterinary Science, The University of Queensland, Building 82, Slip Road, St Lucia, Brisbane, Queensland 4072, Australia.

E-mail: g.milinovich@uq.edu.au

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the stomach and small intestine to the caecum and colon where microbial digestion occurs. The introduction of high levels of fructans results in large-scale changes in the composition of the hindgut microbiota. Using an oligofructose-induction model (van Eps and Pollitt, 2006), we demonstrated a rapid proliferation of oligofructose-utilizing streptococci of the *Streptococcus bovis/equinus* complex immediately prior to the onset of laminitis. This was followed by an equally rapid decline in streptococci as lactobacilli and *Enterobacteriaceae* became more predominant in the post-laminitis period (Milinovich *et al.*, 2006, 2007). These changes mirrored a drop in caecal pH from between 6.2 and 6.8–4.5 or below. However, these studies were either culture-based (Milinovich *et al.*, 2006) or included large culture-dependant elements (Milinovich *et al.*, 2007) and have to be tempered by the knowledge that only 30% of the equine caecum microbiota can be cultivated (Mackie and Wilkins, 1988).

The purpose of the current study was to identify and quantify the key bacterial species in the equine caecum during oligofructose-induced laminitis using a culture-independent approach and to correlate the changes in the major groups of microorganisms with substrates and end products detectable in caecal fluid. Laminitis was induced by nasogastric administration of a bolus dose of oligofructose in five horses fitted with caecal cannulae (van Eps and Pollitt, 2006; Milinovich *et al.*, 2007). Caecal fluid samples collected over a 36 h period were assayed by denaturing gradient gel electrophoresis (DGGE) of PCR amplified V2/V3 variable regions of 16S rRNA genes. Quantitative real-time PCR (qPCR) of the 16S rRNA genes of selected microorganisms further characterized the DGGE-determined microbial community. Microbial changes established by these latter methods were compared with changes in caecal fluid lactate, volatile fatty acid (VFA) and concentrations of fructo-oligosaccharide with degrees of polymerization (DP) of 3–7.

Materials and methods

Laminitis induction, sample collection and processing

This project was approved by The University of Queensland Animal Ethics Committee (SVS/727/04). All animals were continuously monitored by the investigators and the horses were inspected by the Consultant Veterinary Officer to the Animal Welfare Unit at the University of Queensland at the request of the Animal Ethics Committee. If pain could not be alleviated promptly, early euthanasia, even if this was prior to the planned conclusion of the experiment, was a proviso of the protocol.

Details of the five horses (1–5) fitted with cannulae into surgically placed caecal fistulas have been provided in previous publications (Croser and Pollitt, 2006; Milinovich *et al.*, 2007). Laminitis induction was achieved by administration of

10 g kg⁻¹ body weight of oligofructose (Raftilose P95, Orafit Active Food Ingredients, Tienen, Belgium) by a nasogastric tube (van Eps and Pollitt, 2006).

Caecal fluid specimens were collected through the caecal cannulae at 2 h intervals in the 0–24 h post-oligofructose administration (POA) period and at 4 h intervals in the 24–36 h POA period. Specimens were immediately fixed in both 98% ethanol and 4% paraformaldehyde for fluorescence *in situ* hybridization (FISH; Amann, 1995) and frozen at –80 °C for DGGE, qPCR and determination of lactate, VFA and individual (DP3–7) fructo-oligosaccharide concentrations. Additionally, six serial hoof biopsies were collected from each horse at 0, 12, 18, 24, 30 and 36 h POA to monitor histopathological changes consistent with the onset of laminitis (Croser and Pollitt, 2006).

DNA extraction procedure

Caecal fluid samples stored at –80 °C were available for all time points except 14 and 28 h POA from horse 2. DNA for use in DGGE and qPCR was extracted from 1 g (samples 0 h POA from horses 2, 4 and 5 and samples 0, 20, 28, 32 and 36 h from horse 1) or 1 ml (all other samples) of caecal fluid by the repeated bead-beating, column extraction method (Yu and Forster, 2005) and eluted to a final volume of 500 µl. Samples of 1 g were used when the water content was too low to measure the sample volumetrically.

PCR amplification and denaturing gradient gel electrophoresis

PCRs used extracted DNA as templates and the 341f-GC (5'-CGCCCGCCGCGCGCGGGCGGGGCGGGGCGGGGCGGCACGGGGGGCCTACGGGAGGCAGCAG-3') and 534r (5'-ATTACCGCGGCTGCTGG-3') bacterial primers and cycling conditions of the authors (Muyzer *et al.*, 1993) to amplify the variable V2/V3 region of bacterial 16S rRNA genes. All PCRs for DGGE used Red Hot DNA polymerase (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. Amplicons were separated by DGGE (DCode System; Bio-Rad, Hercules, CA, USA) on 8% acrylamide gels with 30–60% formamide/urea gradients. All gels were run at 100 V and 60 °C over 18 h in 0.5 × TAE buffer (Tris-acetate, 0.04 M; EDTA, 0.001 M). A reference ladder of Klieve *et al.* (2007), consisting of amplicons from isolates with various melting profiles spanning the length of the gel, was run on all gels. DNA bands were visualized following electrophoresis by silver staining (Kocherginskaya *et al.*, 2005).

Clone library preparation

Near full-length 16S rRNA gene clone libraries were produced from the 0, 10 and 28 h POA samples from

horses 1 and 4. The 16S rRNA genes were amplified using the universal bacterial primers 27f (5'-AGA GTTTGATCMTGGCTCAG-3') and 1525r (5'-AAGG AGGTGWTCCARCC-3'; Lane, 1991) according to previously reported cycle parameters (Ouwerkerk and Klieve, 2001). All PCRs used Red Hot DNA polymerase (Thermo Fisher Scientific) according to the manufacturer's instructions. Amplificates were cloned using the Topo TA cloning kit (Invitrogen, Carlsbad, CA, USA) and the manufacturer's instructions. The inserts from 16 clones, from each library, were analysed by DGGE as described above.

DGGE band identification

Five additional DGGE ladders were prepared by PCR, using the 341f-GC and 534r primers, from previously isolated equine hindgut microorganisms (Milinovich *et al.*, 2006, 2008) and clones produced in this study. Ladder 1 included the equine hindgut streptococcal species (EHSS) isolates and clones and the *Escherichia coli* clones and isolates, ladder 2 *Lactobacillus* isolates and clones, ladder 3 *Anaerovibrio*-like and *Succinivibrio dextrinosolvens*-like clones, ladder 4 previously isolated *Enterococcus* isolates and *Clostridium*-like clones and ladder 5 other miscellaneous clones including *Actinobacillus rossii*, *Eubacterium rectale* and uncultured microorganisms most closely related to *Anaerostipes* spp., *Subdoligranulum* spp., γ -Proteobacteria and *Lachnospiraceae*. DGGE was subsequently performed on all caecal fluid samples, as described above, against each of the five ladders allowing presumptive identification of banding patterns of interest.

DNA sequencing and analysis

The inserts of representative clones from each DGGE banding pattern were amplified with 27f and 1525r.

Amplificates were purified with the QIAquick PCR purification kit (Qiagen, Hilden, Germany) and sequenced with primers 27f, 1525r, 907r (Lane, 1991) and 803f (Lane, 1991) using a BigDye Terminator (version 3.1) sequencing kit (Applied Biosystems, Foster City, CA, USA). Phylogenetic analyses of 16S rRNA genes were carried out as previously described (Bjornsson *et al.*, 2002).

Quantitative real-time PCR

qPCR assays were used to enumerate bacterial populations of interest as identified by DGGE. All Taq nuclease assays used RealMasterMix Probe kits (Eppendorf, Hamburg, Germany), SYBR green assays used the SYBR GreenER qPCR SuperMix Universal kit (Invitrogen) and the kit manufacturer's instructions. qPCR primer sets (Table 1) targeting the 16S rRNA genes were used at a final concentration of 900 nM. All qPCRs were 25 μ l and used 5 μ l DNA extracted from caecal fluid (described above) diluted 10^{-1} , as templates. The *Anaerovibrio lipolyticus* (Tajima *et al.*, 2001), *Enterobacteriaceae* (Bartosch *et al.*, 2004), *S. bovis* (Klieve *et al.*, 2003) and *S. dextrinosolvens* (Stevenson and Weimer, 2007) qPCR assays used the methods in the publications.

The bacterial assay described by Nadkarni *et al.* (2002) was modified to include a minor groove binder moiety into the probe. This shortened the probe by 10 nucleotides at the 5' end without affecting the T_M and increased the coverage of the probe for bacterial 16S rRNA genes. The original probe (Nadkarni *et al.*, 2002) was identical in sequence to 61.5% of bacterial 16S rRNA sequences in the Ribosomal Database Project (RDP) database, whereas the redesigned probe was identical to 75.9% of RDP database sequences. The cycle parameters for this assay were modified to: an initial step at 94 °C for 1 min, then 40 cycles of 94 °C for 10 s and 60 °C for 10 s.

Table 1 qPCR probes and primers used in this study

Bacterial group targeted	Assay type	Amplicon size (bp)	Forward primer (5'-3')	Reverse primer (5'-3')	Probe (5'-3')	References
All Bacteria	Taq nuclease	466	TCCTACGGGAGG CAGCAGT	GACTACCAGGTA TCTAATCCTGTT	6FAM-CGGCTGCTGGCAC-MGBBHQ	Modification of bacterial assay (Nadkarni <i>et al.</i> , 2002) (Klieve <i>et al.</i> , 2003)
<i>Streptococcus bovis</i>	Taq nuclease	90	ATGTTAGATGCTTGA AAGGAGCAA	CGCCTTGGTGAGC CGTTA	6FAM-CTCACCACACT AGCTAATACA ACGCAGGTCCA-TAMRA	Modification of <i>S. bovis</i> assay (Klieve <i>et al.</i> , 2003)
Equine hindgut streptococcal species (excluding <i>Streptococcus gallolyticus</i>)	Taq nuclease	127	GAAACGATAGCTAA TACCGCATAA	CGCCTTGGTGAGC CKTTA	6FAM-CTCACCACACTAG CTAATACAACGCAGG TCCA-TAMRA	Modification of <i>S. bovis</i> assay (Klieve <i>et al.</i> , 2003)
<i>Enterobacteriaceae</i>	SYBR green	195	AGCAGTAGGGAAT CTCCA	ATTYCACCGCTA CACATG	Not applicable	(Bartosch <i>et al.</i> , 2004)
<i>Succinivibrio dextrinosolvens</i>	SYBR green	73	CGTCAGCTCGTGTC GTGAGA	CCGCTGGCAAC AAAGG	Not applicable	(Stevenson and Weimer, 2007)
<i>Anaerovibrio lipolyticus</i>	SYBR green	597	TGGGTGTTAGAA ATGGATTC	CTCTCCTGCACTCA AGAATT	Not applicable	(Tajima <i>et al.</i> , 2001)
<i>Anaerovibrio</i> and <i>Anaerovibrio</i> -like clones	SYBR green	169	CTCACAGGAGA CTGCCGC	AGCCTGCAATCCG AACTG	Not applicable	This study

The *S. bovis* qPCR assay of Klieve *et al.* (2003) was modified to target all previously isolated EHSS, with the exception of *Streptococcus gallolyticus* subsp. *gallolyticus*, *S. gallolyticus* subsp. *macedonicus*, *Streptococcus henryi* and *Streptococcus caballi* (Milinovich *et al.*, 2006, 2008) and all streptococcal clones with the exception of one clone (H1-28-16). According to BLAST, this clone was most closely related to *S. gallolyticus* subsp. *macedonicus* (1469 nt; 96%). The modified assay did not target any non-streptococcal isolates or clones. The cycle parameters for this assay were identical to the *S. bovis* qPCR assay (Klieve *et al.*, 2003).

The *A. lipolyticus* qPCR assay (Tajima *et al.*, 2001) did not target the *Anaerovibrio*-like clones produced by this study. Therefore, an assay for *Anaerovibrio* and similar clones, but no organisms outside the *Anaerovibrio* genus was designed. The cycle parameters for this assay were: an initial step at 95 °C for 10 min, then 45 cycles of 95 °C for 15 s and 60 °C for 60 s.

Standards for qPCR were produced using *E. coli* (ATCC 25922), *S. bovis* (isolate AR3; Klieve *et al.*, 1989), *A. lipolyticus* (DSM 3074) and *S. dextrinosolvens* (DSM 3072). Briefly, cell counts from overnight cultures were conducted (Ouwerkerk *et al.*, 2002) and concentrations adjusted to 10¹⁰ cells per ml. DNA was extracted (described above) from 1 ml of each 10-fold serial dilution from 10¹⁰ through to 10³ cells per ml. Standards of 5 µl, diluted 10⁻¹, were used as templates in all qPCR assays to allow copy numbers of the targeted genes to be converted to cell numbers. *E. coli* (ATCC 25922) was used as a standard for the total bacteria assay.

Oligonucleotide probe design and optimization

The FISH probes Succ654 (targeting *S. dextrinosolvens* and *S. dextrinosolvens*-like clones) and Ana1141 (targeting *A. lipolyticus* and similar clones) were designed using previously published

methods (Hugenholtz *et al.*, 2001, 2002). All probes (Table 2) were synthesized and labelled at the 5' end with the sulfoindocyanine dyes Cy3, Cy5 or fluorescein isothiocyanate (FITC; Thermo Electron Corporation, Ulm, Germany). The optimum formamide concentrations (Table 2) were determined for FISH using a positive (bacteria with 100% sequence identity) and a negative (bacteria with a one base mismatch, where available) control sourced from previous equine laminitis experiments (Al Jassim *et al.*, 2005; Milinovich *et al.*, 2006) or type-culture collections. The optimum formamide concentration was determined for each probe by carrying out FISH at 0%, 10%, 20%, 30%, 40% and 50% formamide. All probe hybridizations were conducted at 46 °C.

Fluorescence in situ hybridization

Fluorescence *in situ* hybridization used CY3-labelled group-specific probes (Table 2) and FITC-labelled EUBMIX with all fixed samples except 2 and 28 h POA from horses 1 and 2, respectively. An Olympus BX50 microscope with an Olympus BX-FLA reflected light fluorescence attachment (Olympus Optical Co., Ltd, Japan), a ×100 1.30 NA UPlanFl oil-immersion objective and U-MF and U-MWG filters were used to visualize FISH-probed samples. The relative abundance of the bacteria was determined by a previously reported subjective scoring method (Milinovich *et al.*, 2007). Subjective scores were: 0%, occasional presence (fewer than one microorganism per field of vision and not possible to quantify), <1%, 1–5%, 6–10% and then 10% increments up to 91–100%.

Lactate, volatile fatty acid and fructo-oligosaccharide analyses

Lactate, VFA and DP3–7 fructo-oligosaccharide concentrations were determined in caecal fluid

Table 2 Fluorescence *in situ* hybridisation oligonucleotides used in this study

Probe	Probe sequence	16S rRNA target site	Specificity	Formamide %	Positive control	Negative control	References
EUB338 ^a	GCTGCCTCCCGTAGGAGT	338–355	Most but not all bacteria	0–50	NA	NA	(Stahl and Amann, 1991)
EUB338-II ^a	GCAGCCACCCGTAGGTGT	338–355	Bacterial groups not covered by EUB338 and EUB338-III	0–50	NA	NA	(Daims <i>et al.</i> , 1999)
EUB338-III ^a	GCTGCCACCCGTAGGTGT	338–355	Bacterial groups not covered by EUB338 and EUB338-II	0–50	NA	NA	(Daims <i>et al.</i> , 1999)
Ana1141	CGCGGCAGTCTCCTGTGA GTTCCCA	1141–1165	<i>Anaerovibrio lipolyticus</i> and <i>Anaerovibrio</i> -like clones	50	<i>Anaerovibrio lipolyticus</i> (DSM3074)	<i>Mitsuokella jalaludinii</i> (RA2107) ^b	This study
Succ654	TCTCCCCCTGCAATACTC	654–671	<i>Succinivibrio dextrinosolvens</i> and similar clones	20	<i>Succinivibrio dextrinosolvens</i> (DSM3072)	<i>Rhodococcus equi</i> (B06/0148) ^c	This study

Abbreviation: NA, not applicable.

^aEUBMIX was equal amounts of EUB338, EUB338-II and EUB338-III.

^bIsolate from a previous equine laminitis trial (Al Jassim *et al.*, 2005).

^cClinical isolate from The University of Queensland Veterinary Diagnostic Bacteriology Laboratory.

samples. High performance liquid chromatography (HPLC) was used to analyse for lactate and fructo-oligosaccharide and gas chromatography was used for VFAs. Briefly, 4 ml of caecal fluid was mixed with 1 ml of 6% phosphoric acid and centrifuged at 16 °C for 10 min at 1000 g. The supernatant of samples for lactate and VFA analysis was passed through a 300 mg C-18 Sep Pak (Alltech Associates, Deerfield, IL, USA) and a 0.45 µm filter and stored at 4 °C prior to analysis. The supernatant of samples for fructo-oligosaccharide analysis were passed through a GF/B glass fibre filter (Whatman, Maidstone, UK), through 500 mg of Dowex 50 × 8 (H⁺ form; Sigma-Aldrich Co., Buchs, Switzerland) coupled to a 300 mg C-18 Sep Pak (Alltech) and a 0.45 µm filter and stored at 4 °C prior to analysis.

Lactate was determined on an Agilent 1100 HPLC (Agilent Technologies, Palo Alto, CA, USA) by the method of Ewaschuk *et al.* (2002). Samples were passed through a Prevail Organic Acid 5u 250 × 4.6 mm column (Alltech) at a flow rate of 1.0 ml min⁻¹. Mobile phases A and B were 25 mM K₂HPO₄ (pH 2.5) and methanol, respectively. The column was equilibrated in 100% mobile phase A and the gradient was 0–4 min 100% A, 4–10 min linear gradient to 10% B, 10–12 min linear gradient to 25% B, 12–24 min 25% B, 14–18 min linear gradient to 100% A and 18–25 min 100% A. Detection was with a ultraviolet detector at 210 nm calibrated with 50, 100, 200 and 300 µg ml⁻¹ lactate standards prepared from lithium lactate.

VFAs were determined with a Shimadzu GC-17A gas chromatograph (Shimadzu Corporation, Kyoto, Japan) fitted with a splitless injection port glass liner (SGE Analytical Science, Austin, TX, USA) and a capillary column (DB-FFAP 30 m × 0.53 mm × 1.0 µm column; J&W Scientific, Folsom, USA) with the pre-column end attached to the injection port and a flame ionization detector. The oven temperature was initially set to 110 °C for 4 min and increased to 200 °C at a rate of 15 °C per min. The injector and detector temperatures were 220 and 225 °C, respectively, and high purity helium was used as the carrier gas at a column head pressure of 35 kPa. A multi-acid standard containing 35.1 mM acetate, 8.1 mM propionate, 4.0 mM isobutyrate, 5.1 mM n-butyrate, 3.0 mM isovalerate and 3.1 mM valerate was made and prepared with 6% phosphoric acid as described above for caecal fluid.

For fructo-oligosaccharides, an Agilent 1100 HPLC fitted with a Prevail Carbohydrate ES 5u 250 × 4.6 mm column (Alltech) was used with a flow rate of 1.0 ml min⁻¹. Mobile phases A and B were acetonitrile and Milli-Q deionized water, respectively. The column was equilibrated in 90% A and 10% B and the gradient was 0–5 min, 10–25% B; 5–20 min linear gradient, 25–45% B; 20–22 min, 45% B; 22–25 min linear gradient to 10% B and 25–30 min 10% B. Detection was by an Alltech ELSD 2000 detector. Calibration of the ELSD detector was with raffinose at 1.0, 2.0, 5.0 and 10.0 mg ml⁻¹.

Retention times for fructo-oligosaccharides of interest were determined by running a 10.0 mg ml⁻¹ oligofructose (Raftilose P95; Orafit Active Food Ingredients) standard.

Results

Induction of laminitis

As previously reported (Croser and Pollitt, 2006; Milinovich *et al.*, 2007), all horses exhibited depressed appetite by 8 h POA and developed profuse, watery diarrhoea by 16 h POA. All five horses developed acute caecal acidosis with pH dropping from 6.2–6.8 to below 5 by 6–12 h POA and all caecal fluid samples collected between 2 and 24 h POA had pH values significantly lower than the time zero value ($P < 0.05$; Milinovich *et al.*, 2007). Laminitis was diagnosed clinically by the onset of lameness, characteristic of laminitis, in all horses (van Eps and Pollitt, 2004). Clinical laminitis was initially observed in horses 1, 4 and 5 at 20–28 h POA and at 38 and 43 h POA in horses 2 and 3, respectively. Histological changes within the foot, notably rounded lamellar epidermal cell nuclei and attenuation of secondary epidermal lamellae, that signal laminitis onset, were first observed at 12 h POA in horses 1 and 4, 18 h POA in horse 5 and 30 h POA in horses 2 and 3 (Croser and Pollitt, 2006).

Denaturing gradient gel electrophoresis and 16S rRNA gene sequencing

DGGE identified a number of trends in caecal bacterial population shifts, occurring throughout the laminitis process, between the five horses. Over the time course, microorganisms represented by amplicons occupying positions towards the bottom of the gel decreased, whereas those represented by bands positioned towards the top of the gel increased (Figure 1, Supplementary Figures 1–5).

Equine hindgut streptococcal species. All EHSS clones ($n = 12$) and isolates collected from previous studies ($n = 100$) formed a single band by DGGE with the exception of *S. henryi* and *S. caballi* (which together formed one band) and one clone from horse 4 (clone H4-10-15). According to near full-length (>1460 nt) 16S rRNA gene sequence identity, 11 of the 12 EHSS clones were closest to *Streptococcus lutetiensis* (AB362710; 99.7–99.8% ($n = 6$), AF104109; 94.8–95% ($n = 2$), AY442813; 99.2–99.6% ($n = 2$), EB163444; 97.5% ($n = 1$)) and one to *S. gallolyticus* subsp. *macedonicus* (AF459413; 96.9%). The clone that formed a unique banding pattern (H4-10-15) was most closely related to *S. lutetiensis* (AB362710; 99.8%). The clone most closely related to *S. gallolyticus* subsp. *macedonicus* grouped with the other EHSS. Banding patterns characteristic of *S. henryi* and *S. caballi* and of clone H4-10-15 were only observed at low intensity in

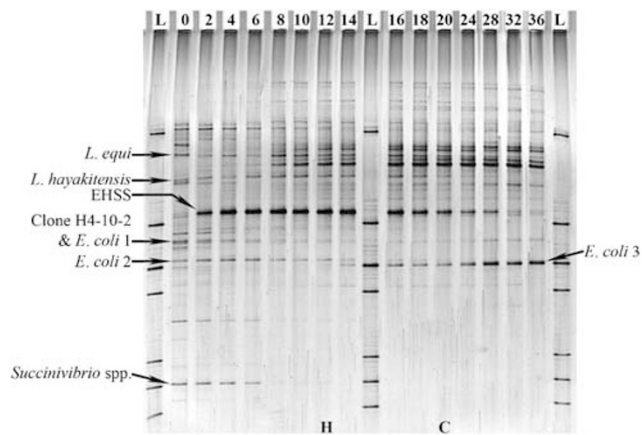


Figure 1 Denaturing gradient gel electrophoresis of the PCR amplified V2/V3 region of 16S rRNA genes from the caecal fluid of horse 4. Numbers at the top of the gel indicate time, in hours post-oligofructose administration (POA), and L=ladder. Horse 4 initially exhibited histological evidence of laminitis (H) at 12 h POA and clinical signs of laminitis (C) at 20 h POA. The microorganisms listed on the left- and right-hand sides of the gel represent the most commonly observed microorganisms from all five horses assayed. Note: BLAST analysis of near full-length 16S rRNA gene sequence data of clone H4-10-2 indicated that this most closely related to *Anaerovibrio lipolyticus* (AB034191: 93.2%).

horses 5 and 4, respectively. The main EHSS band demonstrated a marked and extremely rapid increase in intensity 2–4 h POA (Figure 1) in all horses except horse 2. EHSS of horse 2 initially decreased slightly in intensity before increasing at 16 h POA. The EHSS band was observed at all time points.

Escherichia coli. DGGE of the variable V2/V3 region of the 16S rRNA gene demonstrated a clear and consistent shift in the equine hindgut *E. coli* subpopulations throughout the laminitis process. *E. coli* was found to be represented by two separate bands, in all horses, at the 0 h time point (Figure 1; *E. coli* 1 and 2). These two bands decreased in intensity in all five horses and were not observable in four of the five horses by the final time point analysed at 36 h POA. A third banding pattern, *E. coli* 3, became the most intense of the *E. coli* bands between 16 and 24 h POA in all five horses (Figure 1).

Anaerovibrio. A clone (clone H4-10-2), most closely related to *A. lipolyticus* (AB034191: 93.2%), occupied the same position on the DGGE gel as *E. coli* 1 (Figure 1). Therefore, qPCR and FISH analyses for this microorganism were used to further assess its abundance throughout the laminitis process. Different *Anaerovibrio*-like clones, which formed a second band towards the top of the gel, were observed in two of the five horses.

Succinivibrio. One clone from horse 4 (clone H4-0-8) had highest identity to *S. dextrinosolvens* (Y17600: 96.4%). A DGGE band at the same position as this microorganism was observed, to varying degrees, in all

five horses (Figure 1). This band was typically observed at low to moderate intensity at 0 h POA and intensified slightly between 2 and 8 h POA before becoming non-detectable by 14–20 h POA in horses 1, 3, 4 and 5 and by 32 h in horse 2. The band was most prominent in horse 2, where it peaked in intensity at 6 h POA. Conversely, horse 1 exhibited this band at only very light intensity throughout the laminitis process.

Lactobacilli. The lactobacilli ladder produced in this study (ladder 2) contained lactobacilli isolates and clones which generated eight separate DGGE bands. Of these, only two bands were found to be common in all five horses. According to BLAST of near full-length 16S rRNA gene sequences (>1455 nt) the clones that formed these bands had the highest identity with *Lactobacillus equi* (AB048833: 99.4%) and *Lactobacillus hayakitensis* (AB267406: 99.7%), which are considered to be commensals of the equine gastrointestinal tract (Morotomi *et al.*, 2002; Morita *et al.*, 2007). Intensification of the lactobacilli bands was not as marked as for other species, such as the EHSS and *E. coli* 3, and appeared to be secondary to EHSS proliferation (Figure 1).

Other bacteria. None of the bands from bacterial species in DGGE ladder 5 were consistently observed across the five horses. These microorganisms were, therefore, not considered to be of interest with regard to laminitis, and were not investigated further. Furthermore, no other banding patterns, other than those identified and previously discussed, were observed consistently across all five horses.

Quantitative real-time PCR

Equine hindgut streptococcal species. All qPCR data are supplied in Supplementary Table 1. qPCR results for the total bacteria and the EHSS assay (modified from the *S. bovis* qPCR assay of Klieve *et al.* (2003) to target a wider range of streptococci found in the equine hindgut) are shown in Figure 2. Enumerations of EHSS were, on average, 11 times higher by the modified assay than by the original *S. bovis* assay (data not shown). The EHSS qPCR results correlated with the FISH quantification results with probe Sb127, previously reported for these samples (Figure 2; Milinovich *et al.*, 2007). EHSS constituted $\geq 80\%$ of all bacteria, by qPCR, in three of the five horses prior to the onset of histological signs of laminitis (horses 3–5) and for horses 1 and 2, peaked at 33.0% and 19.1%, respectively. Proportionally, EHSS returned to, or close to, the pre-induction values by 36 h POA in four of the five horses. However, EHSS qPCR copy numbers never resumed their pre-induction values, but remained elevated (Figure 2).

Enterobacteriaceae. The *Enterobacteriaceae* qPCR results were consistent with previously reported

FISH results using the Ent probe (specific for *Enterobacteriaceae*; Milinovich et al., 2007). *Enterobacteriaceae* increased in all horses; this increase

was secondary to the increases in EHSS, was less pronounced and mainly occurred subsequent to the onset of pathological changes in the foot. Furthermore, only two of the five horses harboured *Enterobacteriaceae* in excess of 15% by qPCR (horses 2 and 4).

Succinivibrio. *S. dextrinosolvens* decreased in all horses POA. As the decrease in this microorganism was less pronounced than the decrease in total bacterial numbers, the proportion of *S. dextrinosolvens* increased POA in all horses, except horse 1. The pre-induction proportion of all bacteria that were *S. dextrinosolvens* were 19.4%, 4.0%, 10.1% and 3.7% for horses 2–5, respectively. This value peaked between 2 and 6 h POA, ranging between 8.3% and 40.7% and decreased to below pre-induction levels by 8–10 h POA in horses 3–5 and by 16 h POA for horse 2. *S. dextrinosolvens* in horse 1 was <1% of the total bacteria at all time points.

Anaerovibrio. *A. lipolyticus* (Tajima et al., 2001) never exceeded 0.06% of the total bacterial population by qPCR in any horse, at any time point. However, the broadened *Anaerovibrio* assay developed in this study determined that these bacteria were in excess of 20% in all horses. Pre-induction, *Anaerovibrio* comprised between 3.9% and 12.4% of total bacteria. They increased POA, however, the degree by which they increased varied markedly between horses. Between 4 and 10 h POA these microorganisms comprised 56.2% (6 h POA), >90% (10 h POA), 32.2% (4 h POA), 23.1% (6 h POA) and 61.5% (8 h POA) of the total bacterial population in horses 1–5, respectively. They decreased rapidly in all horses to be at or close to their pre-induction values within 2–12 h of their highest values.

Fluorescence in situ hybridization

Succinivibrio. All FISH data are supplied in Supplementary Table 1. Prior to laminitis induction Succ654-hybridizing bacteria comprised 0%, 31–40%, 6–10%, 21–30% and 11–20% of all bacteria in horses 1–5, respectively. In horses 2–5 they were <1% by 4 h POA and remained at <1% or lower for all time points except 6 h POA for horses 2 and 5 (both 1–5%) and 12 and 16 h POA for horse 2 (21–30%). These bacteria were only observed in the 8 h POA for horse 1

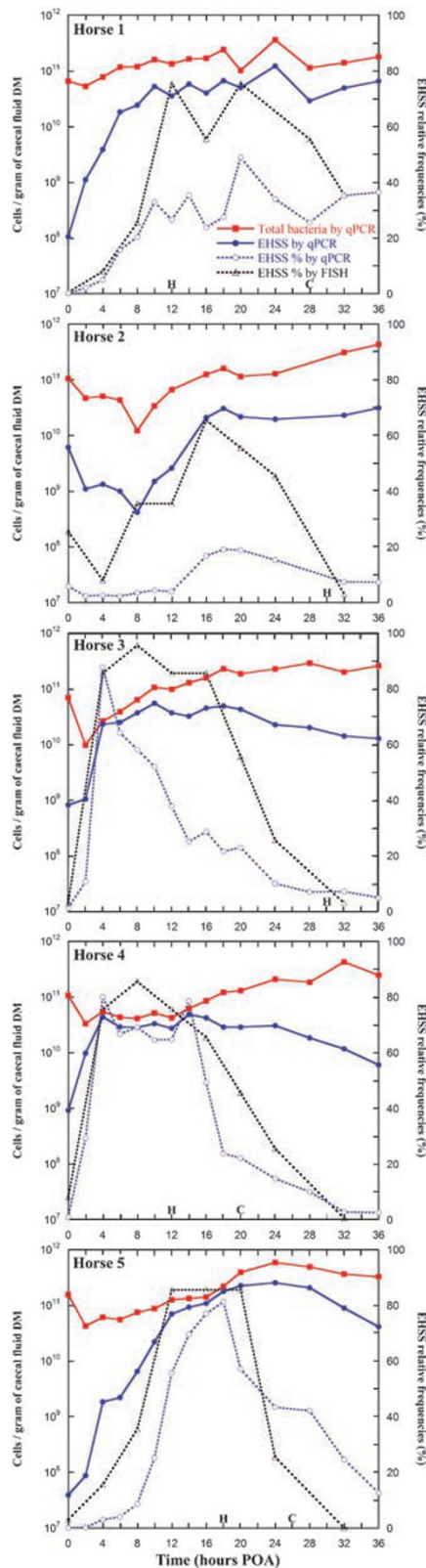


Figure 2 Caecal fluid quantitative real-time PCR (qPCR) results for total bacteria and equine hindgut streptococcal species (EHSS; left y axis) and relative frequencies for Sb127-hybridizing microorganisms by fluorescence *in situ* hybridization (FISH) and EHSS by qPCR (right y axis) for horses 1–5 (top to bottom). The time points at which histological (H) and clinical (C) signs of laminitis were initially observed are indicated above the x axis. Note: horses 2 and 3 were initially observed to exhibit clinical signs of laminitis at 38 and 43 h post-oligofructose administration (POA), respectively.

at fewer than one microorganism per microscope field (occasional).

Anaerovibrio. The *Anaerovibrio*-targeting FISH probe, Ana1141, hybridized to the large, curved Gram-negative rod previously described to increase in relative abundance in both caecal fluid and faeces early in the course of oligofructose-induced equine laminitis (Milinovich *et al.*, 2007). The proportion of bacteria targeted by Ana1141 increased POA in all horses, except horse 4 and mirrored the results of the *Anaerovibrio* qPCR assay, albeit, at slightly lower values. Prior to laminitis induction Ana1141-hybridizing bacteria comprised 6–10%, 31–40%, 1–5%, 11–20% and 1–5% of all bacteria in horses 1–5, respectively. For horses 1–5, respectively, Ana1141-hybridizing bacteria peaked at 41–50% (4 h POA), 81–90% (12 h POA), 6–10% (4 h POA), 11–20% (0 h POA) and 31–40% (8 h POA). Ana1141-hybridizing bacteria decreased to 5% of the total bacterial cell numbers by 20, 32, 8, 16 and 20 h POA for horses 1–5, respectively, and remained at these levels for the remaining times except for 32 and 36 h POA from horse 5 (6–10%).

Lactate, volatile fatty acid and fructo-oligosaccharide analyses

Caecal fluid sucrose and all fructo-oligosaccharides monitored (DP3–7) by HPLC were detected at increased concentrations, in all horses, by 2 h POA (Figure 3). They declined to non-detectable concentrations by 20 h POA in horse 1 and 24 h POA in the remaining four horses. As with the other carbohydrates monitored, caecal fluid fructose concentrations increased within 2 h of induction in four of the five horses. Caecal fluid fructose concentration initially decreased POA in horse 3; however, the caecal fluid fructose concentrations for this horse, and for horse 1, were initially elevated compared with the other three horses (Figure 3). Fructose concentration decreased to, or close to, non-detectable concentrations in all horses by the final time point at 36 h POA.

Caecal fluid lactate and VFA concentrations are displayed in Figure 4. Caecal lactate concentrations immediately increased in all horses except horse 3, which decreased slightly before increasing by 4 h POA. The increase in lactate concentrations occurred concurrently with a decrease in VFAs. VFA concentrations typically increased by the final time

point, 36 h POA. A large, transient increase in acetate, propionate and n-butyrate concentrations was observed at 24 h POA in horse 5.

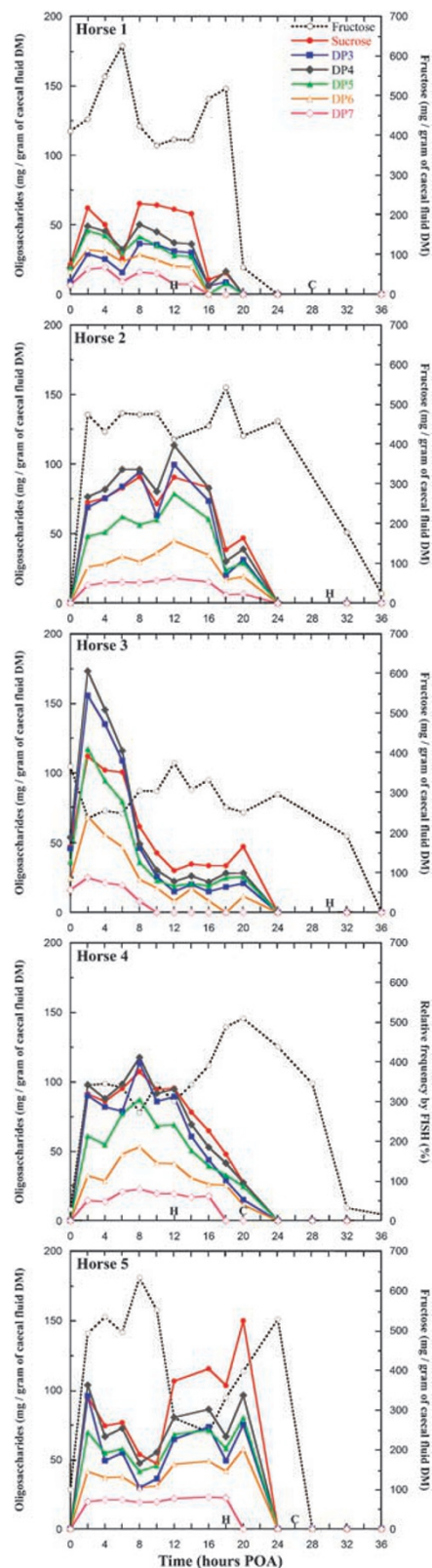


Figure 3 Caecal fluid fructose (right y axis), sucrose (left y axis) and fructo-oligosaccharides, with degrees of polymerization (DP) ranging from 3 to 7, (left y axis) concentrations for horses 1–5 (top to bottom). The time points at which histological (H) and clinical (C) signs of laminitis were initially observed are indicated above the x axis. Note: horses 2 and 3 were initially observed to exhibit clinical signs of laminitis at 38 and 43 h post-oligofructose administration (POA), respectively.

Discussion

Horses are hindgut fermenters and derive 60–70% of their energy from VFAs, produced through microbial fermentation of soluble sugars and structural carbohydrates within the hindgut, which are absorbed across the epithelia of the large intestine (Daly *et al.*, 2001). Thus, VFA concentrations can be used as indicators of intestinal health in the horse (Berg *et al.*, 2005). Oligofructose, administered to horses at a dose rate reflecting a theoretical maximal daily consumption from temperate grasses growing under exceptional climatic conditions for fructan production (10 g kg^{-1} body weight; Longland and Byrd, 2006; van Eps and Pollitt, 2006), reached the caecum within 2 h, in all horses (Figure 3). Microbial oligofructose fermentation resulted in a marked decrease in caecal VFA concentrations, an increase in caecal lactate concentrations and a decrease in caecal pH (Milinovich *et al.*, 2007). These conditions are known to have numerous detrimental effects in the horse including decreased cellulolytic and hemicellulolytic bacterial cell numbers, decreased fibre digestion rates and inhibition of VFA production and absorption (Hussein *et al.*, 2004). Caecal oligofructose fermentation generated a distinct reduction in bacterial diversity, particularly in organisms represented by amplicons that ran towards the bottom of the DGGE gels, immediately POA (Figure 1, Supplementary Figures 1–5) and there was an initial decrease in total bacterial cell numbers by qPCR in all horses (Figure 2).

Previous culture-based studies (Milinovich *et al.*, 2006, 2007) identified EHSS as the most probable aetiological agent of equine laminitis, resulting from alimentary carbohydrate overload, a significant cause of laminitis (>50% of cases; USDA, 2000). Administration of oligofructose resulted in a marked proliferation of EHSS, resulting in increased caecal fluid lactate concentrations (Figure 4) and a concurrent decrease in caecal pH (Milinovich *et al.*, 2007). In the present study, DGGE and qPCR clearly and consistently demonstrated that EHSS became established as the predominant bacteria in the equine hindgut prior to the onset of laminitis. Culture-based studies indicated *S. lutetiensis* to be the most dominant oligofructose-utilizing organism culturable from the equine hindgut prior to the onset of laminitis (Milinovich *et al.*, 2006). In our study, 11 of 12 streptococcal 16S rRNA gene clones had the closest identity to *S. lutetiensis*. Thus *S. lutetiensis* is both the predominant bacterium and the predominant oligofructose-utilizing microorganism present in the equine hindgut prior to the onset of laminitis. Histological signs of laminitis were observed in all horses before lactate concentrations returned to pre-induction values; however, whether the large quantities of lactate produced from oligofructose fermentation are directly involved in the development of laminitis remains to be investigated. Regardless, these results confirm that the

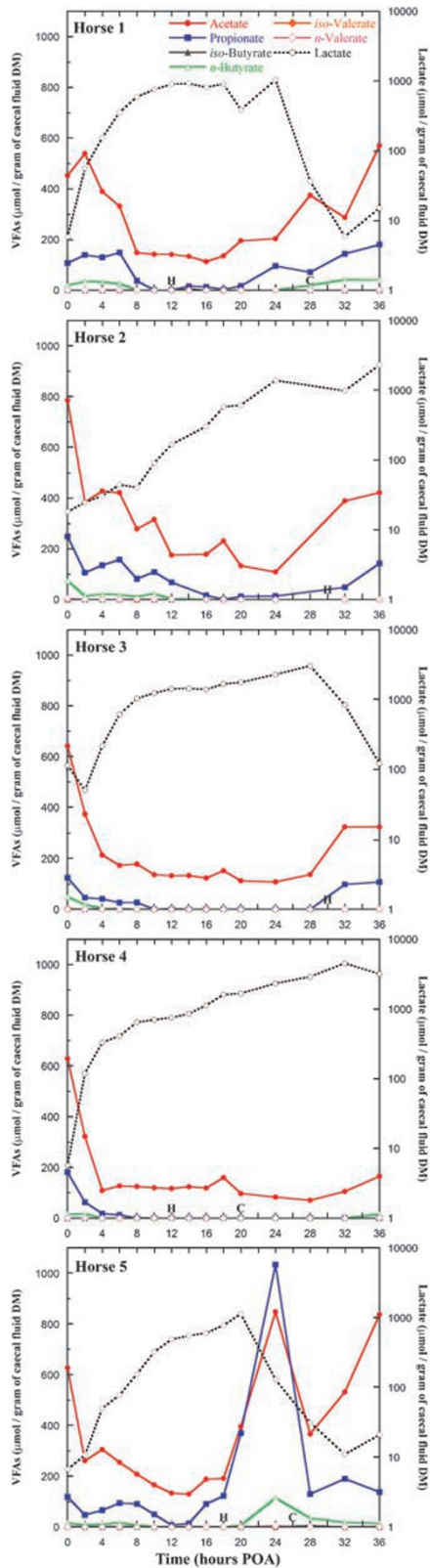


Figure 4 Caecal fluid volatile fatty acid (left y axis) and lactate (right y axis) concentrations for horses 1–5 (top to bottom). The time points at which histological (H) and clinical (C) signs of laminitis were initially observed are indicated above the x axis. Note: horses 2 and 3 were initially observed to exhibit clinical signs of laminitis at 38 and 43 h post-oligofructose administration (POA), respectively.

detrimental effects on hindgut function discussed above are a direct consequence of proliferation of *S. lutetiensis*.

Both qPCR and FISH (with probe Sb127) were used to quantify the proportion of total bacteria that were EHSS in the equine caecum during oligofructose-induced laminitis. Our qPCR targets the 16S rRNA genes in genomic DNA extracts from caecal fluid samples and FISH targets the 16S rRNA in intact whole cells. EHSS qPCR results correlated with FISH relative frequencies throughout the laminitis process (Figure 2). EHSS cell numbers determined by qPCR, however, did not correlate with the FISH results. Results for times up to 16 h POA showed good congruence between qPCR and FISH, demonstrating that these microorganisms increased sharply and then reached a plateau. However, there was no correlation between qPCR and FISH quantification after 16 h POA. FISH demonstrated a rapid decline in EHSS numbers whereas qPCR quantifications remained at the plateau level until 36 h POA. FISH is reported to be sensitive to 10^6 bacterial cells per gram of faeces (Zoetendal *et al.*, 2004). At 32 h POA qPCR detected between 2.97×10^8 and 2.95×10^9 EHSS cells per gram of caecal fluid (values not adjusted for dry matter content) in horses 2–5, values approaching 3000 times the lower detection limits of FISH. Despite qPCR indicating that EHSS were well above the lower detection limits, only a few cells were observed by FISH. One explanation for the lack of congruence between qPCR and FISH may be that the EHSS are being lysed, releasing genomic DNA and other cellular constituents into the caecum. This hypothesis could explain the discrepancy between the qPCR and FISH results. That is, intact cells (observable by FISH) decline, but sufficient genomic DNA fragments remain in the caecum to be detected by qPCR. The lysis of an organism, particularly one in such high numbers as the EHSS in the equine hindgut during laminitis induction, could expose the milieu to several potentially toxic cellular constituents. If EHSS are proven to undergo lysis, liberated cellular components may be responsible for initiating laminitis.

As little as 30% of the equine caecal microbiota are culturable (Mackie and Wilkins, 1988) and while culture-based studies have greatly increased our understanding of the laminitis process, the techniques are prone to biases. This is highlighted by the demonstration, by DGGE, in this study of two previously unidentified groups, *Anaerovibrio*-like bacteria and *Succinivibrio* spp., shown to comprise a high proportion of total bacteria in the equine caecum prior to the onset of laminitis or to proliferate immediately POA. DGGE also demonstrated a clear and consistent shift in *E. coli* subpopulations during laminitis, a phenomenon that has not been reported in any previous studies. These results highlight the effectiveness of DGGE in monitoring changes, or distinguishing differences,

in highly complex microbial communities. The DGGE results of this study corroborate results from FISH (Milinovich *et al.*, 2007), which demonstrated lactobacilli to comprise a small proportion of total bacteria in the caecum prior to the development of histological evidence of laminitis. Only two lactobacilli species, *L. equi* and *L. hayakitensis*, were consistently observed by DGGE to inhabit the equine caecum and to proliferate over the time course.

Previous studies (Milinovich *et al.*, 2006, 2007) showed *Enterobacteriaceae* to be present in low numbers prior to the onset of laminitis in the horse, and to increase throughout the laminitis process. This is corroborated by the *Enterobacteriaceae* qPCR results of this study. It has been reported that there may be in excess of 1000 different strains of coliforms present in the equine hindgut (Golomidova *et al.*, 2007), demonstrating that further study, including collection and study of isolates of each of the *E. coli* subpopulations, demonstrated by DGGE, should be done.

We demonstrated that the *A. lipolyticus* qPCR assay of Tajima *et al.* (2001) was of limited efficacy in enumerating *Anaerovibrio*-like organisms in the equine hindgut during laminitis induction. Due to the proportion of *Anaerovibrio*-like sequences in the clone libraries, we developed a novel qPCR assay and FISH probe (Ana1141) specific for these bacteria. The novel qPCR detected cell numbers as high as 6.56×10^{10} cells per gram of caecal fluid dry matter. Therefore the unidentified, large, curved, Gram-negative rod observed by FISH and described in a previous study (Milinovich *et al.*, 2007) is likely not *A. lipolyticus*, but probably, an *Anaerovibrio*-like bacterium. The 16S rRNA gene sequences obtained from clones targeted in FISH and qPCR are between 87.2% and 93.2% identical to nearest sequences, demonstrating that if the *Anaerovibrio*-like bacteria are homogeneous, they are likely to represent a novel genus and species. The role of these bacteria in laminitis can be definitively determined when pure cultures are available.

According to FISH with the Succ654 probe, *S. dextrinosolvens*-like bacteria were present to varying degrees in all horses prior to administration of oligofructose but declined by 4 h POA and remained low. qPCR substantiated this; however, based on the current data the role of *S. dextrinosolvens*-like bacteria in the laminitis development process cannot be conclusively determined.

Oligofructose is marketed as a dietary supplement with pre-biotic properties for humans and numerous species of animals, including horses. Of the limited publications on the effects of oligofructose on colonic bacterial populations in horses and other species (Beynen *et al.*, 2002; Macfarlane *et al.*, 2006; Milinovich *et al.*, 2007; Respondek *et al.*, 2008), all report a significant increase in streptococci. If EHSS are responsible for laminitis, as we suggest, the effect of dietary supplementation with oligofructose

at pre-biotic dosage rates needs to be studied promptly.

Since the development of the first experimental model for inducing laminitis of alimentary origin (Garner *et al.*, 1975), numerous species of bacteria have been implicated as possible aetiological agents. However, substantial evidence demonstrates EHSS to be the most likely causative agent. The establishment of this group of organisms as aetiological agent of equine laminitis cannot occur conclusively without determining the sequence of events that culminate in lamellar separation. The results of this study suggest EHSS undergo a lytic event and liberated cellular components may constitute the laminitis trigger factor.

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