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# A liquid chromatography–tandem mass spectrometry-based investigation of the lamellar interstitial metabolome in healthy horses and during experimental laminitis induction

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**Highlights for Review**

- Metabolomic analysis of lamellar dialysate can differentiate horses developing experimental sepsis-associated laminitis from controls.
- Malate, pyruvate, aconitate and glycolate in lamellar dialysate, and malate alone in plasma, were identified as the source of differentiation.
- Changes in energy metabolism intermediates in the lamellar interstitium occurred during laminitis development and were not present in plasma.
- Further investigation of local bioenergetic failure as a cause of laminitis due to sepsis is warranted.
- Open profiling could further assess changes to the metabolome elicited by laminitis.


## Abstract

Lamellar bioenergetic failure is thought to contribute to laminitis pathogenesis but current knowledge of lamellar bioenergetic physiology is limited. Metabolomic analysis (MA) can systematically profile multiple metabolites. Applied to lamellar microdialysis samples (dialysate), lamellar bioenergetic changes during laminitis (the laminitis metabolome) can be characterised. The objectives of this study were to develop a technique for targeted MA of lamellar and skin dialysate in normal horses, and to compare the lamellar and plasma metabolomic profiles of normal horses with those from horses developing experimentally induced laminitis. Archived lamellar and skin dialysate ($n$=7) and tissue ($n$=6) from normal horses, and lamellar dialysate and plasma from horses given either 10 g/kg oligofructose (treatment group, OFT; $n$=4) or sham (control group, CON; $n$=4) were analysed. The concentrations of 44 intermediates of central carbon metabolism (CCM) were determined using liquid chromatography – tandem mass spectrometry. Data were analysed using multivariate (MVA) and univariate (UVA) analysis methods.

The plasma metabolome appeared to be more variable than the lamellar metabolome by MVA, driven by malate, pyruvate, aconitate and glycolate. In lamellar dialysate, these metabolites decreased in OFT horses at the later time points. Plasma malate was markedly increased after 6 h in OFT horses. Plasma malate concentrations between OFT and CON at this time point were significantly different by UVA. MA of lamellar CCM was capable of

differentiating horses developing experimental laminitis from controls. Lamellar malate, pyruvate, aconitate and glycolate, and plasma malate alone were identified as the source of differentiation between OFT and CON groups. These results highlighted clear discriminators between OFT and CON horses, suggesting that changes in energy metabolism occur locally in the lamellar tissue during laminitis development. The biological significance of these alterations requires further investigation.

*Keywords:* Bioenergetic; Chromatography; Energy metabolism; Metabolomic; Sepsis.

## Introduction

Failure of lamellar energy metabolism (bioenergetic failure) is thought to be involved in the pathophysiology of sepsis-related (Belknap et al., 2009), endocrinopathic (French and Pollitt, 2004; Pass et al., 1998) and supporting limb (van Eps et al., 2010) laminitis in the horse. High lamellar glucose consumption has been demonstrated both in vitro (Pass et al., 1998) and in vivo (Wattle and Pollitt, 2004). Lamellar hemidesmosomes, specialised multiprotein complexes that participate in the attachment of epithelial cells to their underlying basement membrane (Borradori and Sunnenberg, 1999), are lost in the absence of glucose and the dermo-epidermal attachment becomes weak (French and Pollitt, 2004; Pass et al., 1998). It has recently been proposed that sepsis-related laminitis may also occur secondary to inappropriate utilisation of energy substrates (i.e. glucose) (Belknap et al., 2009), as is thought to occur in sepsis-related organ dysfunction in humans (Callahan and Supinski, 2005; Fullerton and Singer, 2011; Leverve, 2007; Singer, 2008). Despite the purported role of energy failure in laminitis pathogenesis, current knowledge about lamellar bioenergetic physiology is limited.

The goal of metabolomic analysis (MA) is the comprehensive measurement of metabolite concentrations associated with a biological system at the cellular, tissue or whole organism level (Goodacre et al., 2004). MA results in the systematic profiling of multiple metabolites and their temporal (or population) changes in biofluids and tissues (Oliver et al., 1998). It can be undertaken using two broadly different approaches, namely, open (or untargeted) profiling metabolomics or targeted (quantitative) metabolomics (Goodacre et al., 2004). Targeted metabolomics in veterinary science can be regarded as an extension of a clinical chemistry panel whereby several predetermined analytes are quantitatively measured to assess their role in an underlying process. A recent open metabolomics study of serum from horses before and after experimentally-induced laminitis revealed evidence of dysregulation

of fatty acid metabolism, accumulation of organic acids (lactate) and identified that the amino acid citrulline decreased in serum prior to the onset of clinical laminitis (foot pain) (Steelman et al., 2014).

Using tissue microdialysis, our laboratory has examined the major energy metabolites (glucose, lactate and pyruvate) in the interstitium of the lamellar and skin dermis in normal horses over 24 h (Medina-Torres et al., 2014). In the current study we sought to combine tissue microdialysis and metabolomics to examine the changes in lamellar energy central carbon metabolism that occur locally during the development of laminitis. The analysis of the three major energy metabolites previously described (Medina-Torres et al., 2014) was considerably extended in this study by measuring the major energy metabolite pathway intermediates of glycolysis, the pentose phosphate pathway and the Krebs cycle, as well as nucleotides and co-factors. The primary aim of this MA was to determine the source and/or metabolic sequelae of any perturbation caused by experimental induction of laminitis and subsequent bioenergetic failure. Our objectives were: (1) to develop a reliable and reproducible technique for MA of equine lamellar and skin microdialysis samples (dialysates) and tissue samples using high performance liquid chromatography (HPLC) coupled to tandem mass spectrometry (MS/MS); (2) to compare dialysate and tissue extracts of lamellar tissue with the skin using targeted (quantitative) MA, and (3) to use targeted metabolomics to compare serial lamellar and plasma samples from normal horses with those of horses undergoing laminitis induction using the oligofructose (OF) model.

## Materials and methods

### *Samples*

Archived (< 1 year at -80 °C) samples from adult Standardbred horses were used for analysis. Samples were collected during previous studies approved by The University of

Queensland Animal Ethics Committee (AEC) that monitors compliance with the Animal Welfare Act (2001) and the Code of Practice for the care and use of animals for scientific purposes (current edition) (approval numbers SVS/257/11/GJRF of 6October 2011 and SVS/338/11/UQ of 13 January 2012).

Lamellar and skin microdialysis samples were obtained every 2 h over a 24 h study period from seven healthy horses; skin and lamellar tissue blocks were harvested from six healthy horses immediately after euthanasia, and lamellar microdialysis and plasma samples were obtained from eight horses at 0, 6, 12 and 24 h after nasogastric administration of either a bolus dose of 10 g/kg OF (Invita) (treatment group, OFT; *n*=4) as previously described (van Eps and Pollitt, 2006), or sham treatment (control group, CON; *n*=4).

The OFT horses had developed Obel grade 1 lameness (Obel, 1948) at 18 - 22 h after OF dosing and had histological evidence of acute laminitis at 48 h. Dialysate was collected with coaxial microdialysis probes with a 0.5 × 10 mm, 100 kDa cut-off membrane (CMA20, CMA-Microdialysis), inserted into the lamellar tissue of one forelimb and into the skin dermis at the tail base as previously described (Medina-Torres et al., 2014). All probes were perfused with isotonic, polyionic sterile perfusion fluid (T1, CMA-Microdialysis) containing 40 g/L dextran-70 (Sigma-Aldrich) to prevent perfusate loss (Rosdahl et al., 1997). Plasma was separated by immediate centrifugation (15,000 × *g*, 10 min) of whole blood samples collected into heparinised tubes (Vacuette, Greiner Bio-One) in OFT and CON horses via a 16G indwelling catheter (Mila International) placed aseptically in the right jugular vein.

*Development and optimization of LC-MS/MS*

In each sample, central carbon metabolism (CCM) was assessed by liquid chromatography - tandem mass spectrometry (LC-MS/MS) using a targeted method. For

lamellar and skin microdialysis samples, assessment of the perfusion fluid (perfusate) was performed to determine its suitability as a calibration matrix. The performance of a perfusate-based calibration was then measured and compared against an aqueous 'blank' calibration through standard addition of the metabolite panel (reference standards; Sigma-Aldrich) to the perfusate matrix. Thereafter lamellar and skin microdialysis samples were measured to assess the need for further optimization based upon sample-related effects on the analytical system.

To analyse the metabolite composition of lamellar and skin dermis tissue sections, metabolites were extracted using a procedure adapted from Want et al. (2013). In brief, 50 mg of each sample were sliced with a scalpel, suspended in 2 mL of 1:1 methanol:purified water plus 2 µL 1 mM azidothymidine (AZT) as an internal quantitative standard, and homogenised for 5 min at 20,000 rpm using an Omni TH Homogenizer (Omni International). Purified water was generated using a water purification unit (Elga Purelab Classic; Veolia Water Solutions and Technologies). The fluid fraction/supernatant was collected and centrifuged for 15 min at 13,000 *g* at 4 °C. The supernatant (800 µL) was retrieved and chloroform (800 µL) added before vortexing for 15 s. Samples were then centrifuged for 10 min at 13,000 *g* at 4 °C and the supernatant (upper polar phase; 600 µL) collected. Addition of chloroform (600 µL), vortexing and centrifugation were repeated. The upper polar phase was collected and dried down in a vacuum centrifuge (Eppendorf Concentrator Plus) for 180 min at 45 °C using the V-AQ program. The dried sample was resuspended immediately in 100 µL of purified water for LC/MS-MS analysis. Plasma (50 µL) was processed using the same extraction procedures, without the need for the initial tissue homogenization step.

LC-MS/MS data were acquired using a Dionex UltiMate 3000 LC system coupled to an ABSciex 4000 QTRAP mass spectrometer as described by Dietmair et al. (2012), with the following modifications: the analytical column was equipped with a pre-column Security

Guard Gemini-NX C18 4 × 2 mm I.D. cartridge (Phenomenex) and five additional analytes were quantified, as well as AZT (internal standard). The LC system was controlled by Chromeleon software v6.80$^{n}$, and chromatographic separation achieved by injecting 10 µL onto a Gemini-NX C18 150 × 2 mm I.D., 3 µm 110 Å particle column (Phenomenex). The column oven temperature was controlled and maintained at 55 °C throughout the acquisition and the mobile phases (adapted from Luo et al., 2007), were as follows: 7.5 mM aqueous tributylamine (puriss plus grade; Sigma-Aldrich) adjusted to pH 4.95 (±0.05) with glacial AR-grade acetic acid (eluent A; Labscan) and LC-grade acetonitrile (eluent B; Labscan). The mobile phase flow rate was maintained at 300 µL/min throughout the gradient profile (Table 1), and introduced directly into the MS with no split.

The MS was equipped with a TurboV electrospray source operated in negative ionisation mode, and data acquisition controlled by Analyst v1.5.2 software (ABSciex). The following optimised parameters were used to acquire scheduled Multiple Reaction Monitoring (sMRM) data: ion spray voltage −4500V, nebulizer (GS1), auxiliary (GS2), curtain (CUR) and collision (CAD) gases were 60, 60, 20 and medium (arbitrary units), respectively, generated in a N300DR nitrogen generator (Peak Scientific). The auxiliary gas temperature was maintained at 350 °C.

A total of 44 analytes were quantified using HPLC-MS/MS; the analytes and analyte-dependent parameters for the detection of central carbon metabolites are presented in Supplementary Table 1. For all analytes the entrance potential (EP) was -10 volts. Samples were run with sample- and analyte-relevant calibration standards and pooled quality control samples (Hodson et al., 2009; Sangster et al., 2006) to control for reproducibility of data acquisition and to ensure data integrity. The order of acquisition of the samples was randomised to remove/minimise any bias or batch effects related to sample type or, if

applicable, treatment regimes. Analyte stock solutions were prepared in purified water, and aliquots of each solution were mixed to achieve a final calibrant solution at 200 μM. This calibrant solution was diluted to provide a total of 20 calibration standards at 200, 150, 100, 90, 70, 50, 40, 25, 12.5 and 1:1 serial dilutions to 0.006 μM, constituting $7 \leq x \leq 20$ calibration points for all analytes to account for differential responses in the mass spectrometer.

*Data processing and statistical analysis*

Data acquired by HPLC-MS/MS were processed using MultiQuant v2.1.1 software (ABSciex). Multivariate analysis (MVA) of the data was performed using SIMCA v13.0.3.0 software (MKS Umetrics). Two MVA methods were applied, namely principal component analysis (PCA) and orthogonal projection to latent structures-discriminant analysis (OPLS-DA). For more information relating to these methods see Trygg et al. (2007) and Bylesjö et al. (2006).

In normal horses, results were compared between lamellar and skin dermis dialysate, and between lamellar dialysate samples and tissue sections. In the OF study animals, results were compared between lamellar dialysate from OFT and CON groups, between time points within each sample group, and between lamellar dialysate and plasma. Using GraphPad Prism v6.00 for Windows, univariate analysis (UVA) was then performed on selected metabolites identified by MVA. The data were tested for normality using D'Agostino-Pearson omnibus K2 normality tests; non-parametric tests were applied if the data failed this test. Metabolite concentrations in lamellar dialysate and plasma were compared at each time point between OFT vs. CON horses. Significance was set at $P < 0.05$.

**Results**

There was no evidence to suggest that, in comparison to a water-based calibration, the T1 solution with 40 g/L dextran-70 matrix caused any overt suppression or enhancement of the MS detection of analytes from CCM. Lamellar and skin microdialysis samples were subsequently measured successfully after extraction, so no further processing of the samples was required. The metabolite composition of both lamellar and skin tissue sections was also successfully determined; without extensive testing of multiple methods the extraction protocol was deemed suitable for characterisation of intracellular CCM analytes in these two tissues.

*Skin vs. lamellar dialysate*

Multivariate analysis was used to compare the data obtained from lamellar and skin dialysate, initially using PCA to summarise the major sources of variance in the data. Fig. 1a shows the PCA scores plot for this comparison and it is clear from this plot that the distributions of the samples from the skin and the lamellae were similar and approximately overlap each other. When applying alternative colouring to the PCA (Fig. 1b) based upon horse (subject), some structure to the variance can be observed relating to the horse from which the samples were taken. Supervision of the MVA using OPLS-DA was also unable to clearly separate skin and lamellar samples based upon their detectable metabolome in this experiment (Fig. 1c).

*Lamellar dialysate vs. plasma*

PCA of the metabolome of these samples showed clear differences, as observed in Fig. 2a. In general the plasma metabolome appeared more variable than that of the dialysate. This was driven by central carbon metabolites such as malate, where clear differences were not only observed between plasma and lamellar dialysate but also between the control and treated groups. Marked increases in malate where seen, particularly at the 6 h time point in the plasma of OFT-treated subjects (Fig. 2c). Fig. 2b shows the loadings relating to the scores

plot in Fig. 2a and highlights the variables and therefore the metabolites that are influential in the differentiation between plasma and lamellar dialysate. Clearly observable group- and time-dependent metabolite profile differences were also noted for metabolic intermediates such as aconitate (Fig. 2d), pyruvate (Fig. 2e) and glycolate (Fig. 2f).

UVA of the lamellar dialysate revealed significantly ($P<0.05$) higher malate at the 6 h time point and lower malate and aconitate at the 24 h time point in OFT horses compared with CON (Appendix: Supplementary Fig. 1). Pyruvate and glycolate concentrations were not found to be significantly different between OFT and CON, however a decrease in the concentrations of all four metabolites was apparent in the OFT group at the later time points, while concentrations remained stable in the CON group (Appendix: Supplementary Fig. 1). Plasma malate concentrations increased significantly at the 6 h time point in OFT compared to CON horses (Appendix: Supplementary Fig. 2).

*OFT vs. CON*

After investigating the overall differences between metabolite concentrations in lamellar and plasma samples, a more directed analysis was performed to look specifically at the comparison between lamellar microdialysate samples from CON and OFT subjects, as well as comparing the same for plasma extracts. In the case of plasma extract samples, PCA and OPLS-DA models highlighted malate as the only major difference between the metabolome of the CON and OFT horses in plasma. In the case of the lamellar dialysate, models were generated for all time points other than the zero time point (i.e. before treatment) and therefore metabolomic differences were observed based upon OF treatment (Figs. 3 a-f).

*Lamellar tissue extracts vs. skin tissue extracts*

A comparison of tissue extracts from lamellae and skin was also performed in an attempt to assess the central carbon metabolomic differences between these tissues. Figs. 4a-f show the supervised (OPLS-DA) separation of the two tissue extracts and highlights a number of clear differences in the central carbon metabolite content of these tissues. It should be noted that the extraction procedure was controlled with a generalised internal standard (AZT) to account for losses/reduced recovery, and that the differences observed are not due to a consistent increase of metabolites in one tissue after extraction, since metabolite levels are both higher and lower in one tissue compared to the other. The lamellar extracts were found to be more variable in metabolite content than the skin extracts, as can be observed by the tighter distribution of the skin samples in the scores plot (Fig. 4a).

*Lamellar tissue extracts vs. lamellar dialysate*

The central carbon metabolome of lamellar tissue extracts was compared with that of the lamellar dialysate to ascertain how well the microdialysate metabolite profile reflects the lamellar tissue. Figs. 5a and b show the loadings from unsupervised (PCA) and supervised (OPLS-DA) analyses of the samples ($n$=6/group), with a separation in Fig. 5a clearly resolved by supervision in Fig. 5b. Column plots of four metabolites are shown in Figs. 5c-f to highlight the differences in profile for many of the metabolites, the vast majority of which have much greater concentration in lamellar tissue with the exception of malate (Fig. 5f), succinate and glucose 1-phosphate. A summary of the concentrations of all analytes for the various biofluid and tissue extracts are detailed in the Appendix at Supplementary Table 2.

## Discussion

A method for MA of equine lamellar and skin dialysate and tissue extracts was successfully developed and enabled measurement of the CCM in microdialysate, plasma and tissue extract samples.

Principal component analysis of the data from the lamellar and skin tissue of healthy adult horses demonstrated that, by comparison, lamellar and skin dialysate had a similar metabolite composition (Fig. 1a). When the PCA was coloured by subject (Fig. 1b) a pattern of variance related to the sample source (i.e. the horse) was observed. In general this means that the site of sampling (skin vs. lamellae) was not a major discriminating factor when considering possible differences in metabolome, whereas inter-subject variability showed a degree of influence on the metabolomic content of the tissues. Furthermore, OPLS-DA supervision of the MVA also failed to separate lamellar and skin dialysate samples based upon their CCM (Fig. 1c), meaning there was a degree of similarity in the metabolomic content of microdialysis samples obtained from these two collection sites. As the dialysate from both sites can be expected to contain metabolites that diffuse freely across the membrane, the similarities in dialysate composition demonstrates that in the normal (healthy) horse the interstitial fluid composition of the lamellar tissue is similar to that of the skin.

Clear differences between the metabolome of lamellar dialysate and plasma from CON and OFT subjects were observed with PCA (Fig. 2a). Unexpectedly, the metabolome of the plasma samples was found to be more variable than that of the lamellar dialysate. This finding may seem surprising given the homeostatic control of plasma metabolite concentrations in particular but could possibly be explained by two factors; firstly the plasma sample is a snapshot in time whereas the microdialysate sample is collected over a longer period and as such provides a more ‘averaged signal’ of metabolite concentrations; secondly, and linked to the first, the plasma sample is more likely to be affected by external influences such as feeding immediately prior to sample collection.

The variability in plasma was driven by four central carbon metabolites (i.e. malate, aconitate, pyruvate and glycolate; Figs. 2b-f), which were not only influential in the differentiation between the two tissue sample types, but also provided differentiation between the CON and OFT groups (e.g. significant increase in malate at the 6 h time point in OFT plasma). However, when looking specifically at the comparison between plasma extracts from CON and OFT subjects in isolation, only malate was indicated as a discriminatory metabolite. The time-related profile for malate suggests that the determination of plasma concentrations for this metabolite may be a suitable early predictor of laminitis development (at least in laminitis due to alimentary oligofructose overload), although further investigations with larger subject numbers would be required to confirm its utility. Furthermore, determination of plasma malate concentrations at intermediate time points (e.g. hourly sampling from 0 – 12 h after OF administration) would be necessary to identify the evolution of this rise in malate, the time point when the maximum concentration (peak) is reached, and its duration (i.e. the potential diagnostic window). However, as malate is an intermediate metabolite with important roles in energy producing pathways such as the tricarboxylic acid cycle (TCA) in animals (Alberts et al., 2008) as well as the glyoxylate cycle in bacteria (Kornberg and Krebs, 1957), and the naturally occurring malate molecule (i.e. L-malate) is identical in bacteria and mammals (Alberts et al., 2008), the source of plasma malate in the present study cannot be determined. It could be a by-product of bacterial fermentation of OF in the gastrointestinal tract, which would be consistent with the timing of the increase in plasma in this experiment. If this is the case, the use of malate as a predictor of laminitis in naturally occurring laminitis might be precluded.

Contrary to malate, two different isoforms of lactate occur naturally: L-lactate in mammals and D-lactate in bacteria (Alberts et al., 2008). Thus, determining the plasma D-lactate concentration could help establish if this and other intermediate metabolites (such as

malate) originated in the intestinal lumen as a result of the bacterial disbiosis known to occur after OF administration (Millinovich et al., 2006). However, the LC-MS/MS method used in this study could not distinguish between the two lactate isoforms, and other means of differentiation were not attempted. Furthermore, plasma lactate was not a discriminatory metabolite between the CON and OFT groups. Though the absence of a discriminatory shift in plasma lactate concentrations might indicate that the source of other measured metabolites (including malate) may be other than the intestinal intraluminal bacteria, this cannot be established with our findings in the present study.

Thus, whether the discrimination between the CON and OFT groups observed with malate is attributable to an influx of bacterial metabolites from the intestine or a consequence of metabolic disruption attributable to the development of sepsis-associated laminitis in the horse remains to be determined. This could be an important future step in understanding the pathophysiology of sepsis-associated laminitis and in the identification of potential biomarkers for early disease diagnosis.

MA of lamellar dialysate was capable of differentiating between OFT and CON groups. The analysis highlighted two metabolites (malate and aconitate) as clear discriminators between OFT and CON horses. These results suggest that changes in energy metabolism do occur locally in the lamellar tissue during laminitis development and warrant further investigation using the lamellar microdialysis technique. However, the changes may not be biologically significant as they occurred in the later time points, coinciding with the onset of lameness (18–22 h), and after the developmental phase of the disease. This is further confounded by the fact that microdialysis is an interstitial sample, where the observed changes may not represent the true intracellular metabolic status and could be affected by other processes such as inflammation.

In general, lamellar tissue was found to have higher metabolite concentrations in comparison to lamellar dialysate, with the exception of malate, succinate and glucose 1-phosphate. The extracellular origin of the dialysate dictates that concentrations are likely to be lower than the intracellular pools, as the content of the dialysate consists for the most part of TCA intermediates and pyruvate, all of which are produced in the cellular cytoplasm. Assessing a more comprehensive dialysate metabolome using a larger microdialysis probe pore size and with the inclusion of amino acids, fatty acids and carbohydrates would be recommended for future studies in order to comprehensively examine lamellar metabolism during laminitis development. A similar approach was utilised recently for trapezius myalgia in humans (Hadrevi et al., 2013). Furthermore, concurrent assessment of regulatory enzymes of energy metabolism (glycolytic and oxidative) could help determine if lamellar energy failure is a feature of laminitis in the horse. Phosphofructokinase, a rate limiting enzyme of the glycolytic pathway, as well as electron transport chain components were genetically downregulated in muscle from a rodent sepsis model (Callahan and Supinski, 2005).

The main limitations of our study were the low number of subjects included and that only 4/13 time points collected were assessed in OFT and CON horses (due to financial and logistical restrictions). However, despite these limitations, targeted MA and MVA were successful in differentiating OFT and CON horses and potential plasma/lamellar dialysate biomarkers were identified.

## Conclusions

MA of the lamellar dialysate samples for central carbon metabolites was found to be capable of differentiating horses developing experimental sepsis-associated laminitis from controls in the OF model. Malate, pyruvate, aconitate and glycolate in lamellar dialysate, and

malate alone in plasma, were identified as the source of differentiation between OFT and CON groups. The origin and clinical usefulness of these potential biomarkers for early identification of naturally occurring laminitis remain to be determined. These significant changes in energy metabolism intermediates in the lamellar interstitium occurred during laminitis development and were not present in plasma samples, suggesting that further investigation of local bioenergetic failure as a cause of laminitis due to sepsis is warranted. Having established the central carbon metabolome in these samples the next step would most likely be an open profiling approach to further assess the changes to the metabolome elicited by experimentally-induced laminitis.

## Conflict of interest statement

None of the authors of this paper has a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the paper.

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## Appendix: Supplementary material

Supplementary data associated with this article can be found in the online version at doi: setters please insert doi number

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**Figure Legends:**

**Figure 1**: MVA of skin and lamellar dialysate samples: (a) PCA scores plot of skin (S - blue) and lamellar (L - green) microdialysate showing similar data distributions for each tissue type. Numbers represent time points of collection; (b) PCA scores plot coloured by horse to show that some of the variance explained by the MV model is due to inter-subject (horse) variability; (c) OPLS-DA scores plot to show incomplete supervised separation between the sample metabolomes; (d) bar charts showing selected metabolites fumarate, glycolate, glyoxylate and α-ketoglutarate as an example of similar distributions in skin and lamellar microdialysate.

**Figure 2**: MVA of lamellar dialysate and plasma samples: (a) PCA scores plot of lamellar dialysate (D - green) and plasma (P – blue) showing different data distributions for each tissue type. Letters next to each point represent the subject from which the sample was collected (A-D = oligofructose-treated horses; E-H = controls); (b) loadings plot for (a), highlighting the four variables (red points = metabolites; MAL: malate; PYR: pyruvate; ACO: aconitate; Glycol: glycolate shown in c-f) that were influential in the differentiation between the two tissue sample types. Profiles for (c) malate, (d) aconitate, (e) pyruvate, and (f) glycolate for each horse (X-axis: A – H) at each successive time point (0, 6, 12, 24 h = respectively, green and blue bars from left to right for each horse).

**Figure 3**: Differences in the lamellar dialysate metabolome after oligofructose treatment; OFT – red, CON – green; (a) PCA at the 6 h time point; (b) profile of malate at the 6 h time point; (c) PCA at the 12 h time point; (d) profile of pyruvate at the 12 h time point; (e) PCA at the 24 h time point; (f) profile of aconitate at the 24 h time point.

**Figure 4**: Differences in the metabolome of lamellar extracts (green) and skin extracts (blue); (a) OPLS-DA scores plot of the extracts; (b) loadings plot of the metabolites with 4 highlighted variables (metabolites) in red, each shown in detail in c-f as column plots of (c) citrate; (d) glycolate; (e) UDP-glucose and (f) dihydroxyacetone phosphate. The four highlighted metabolites show a clear difference in central carbon metabolite content, which results in separation of the two tissue extracts.

**Figure 5**: Differences in the metabolome of lamellar dialysate (green) and lamellar tissue extracts (blue); (a) PCA scores plot of the comparison of lamellar dialysate vs. tissue extract; (b) OPLS-DA scores plot of lamellar dialysate vs. tissue extract; c-f column plots of (c) a-ketoglutarate; (d) aconitate; (e) dihydroxyacetone phosphate and (f) malate.

Table 1: Liquid chromatography mobile phase gradient profile.

| Time (min) | Eluent A (%) |
|---|---|
| 0 | 100 |
| 8 | 100 |
| 20 | 80 |
| 30 | 73 |
| 31 | 0 |
| 33 | 0 |
| 34 | 100 |
| 50 | 100 |