final report

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Prepared by: A/Prof Luciano A. Gonzalez, Claudia Blakebrough-Hall and Prof Michael D'Occhio
The University of Sydney

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Experiment 1 - Metabolomics of Bovine Respiratory Disease

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Executive summary

Bovine Respiratory Disease (BRD) is a multifactorial disease complex involving both bacterial and viral pathogens which makes its diagnosis difficult via pathogen identification. It is currently the most significant disease affecting feedlot cattle, causing huge economic and productivity losses to the industry. Current diagnosis methods in feedlots often have low accuracy in detecting sick animals and there is consequently a need to develop alternative diagnostic tools for BRD in feedlots.

The aim of the current study was to develop diagnosis methods for BRD using the blood metabolome profile of steers sampled at a commercial feedlot.

Visually sick (n=148) and visually healthy (n=152) steers were pulled from their pens by pen riders and brought to the hospital shed for clinical assessment and blood sampling for metabolomics analysis. Lung lesions indicative of BRD were scored for all trial animals upon slaughter. Nuclear magnetic resonance (NMR) spectrometry was used to identify chemical features in plasma to predict BRD status using classification and regression trees. Due to a lack of a universal gold standard for BRD diagnosis, six reference diagnosis methods were used to define an animal as sick or healthy: pen rider visual diagnosis (PD), rectal temperature diagnosis (TD; rectal temperature ≥ 40 ºC), lung auscultation diagnosis (LAD; lung auscultation score ≥ 2), hospital diagnosis (HD; sick according to either TD or LAD, or both), feedlot diagnosis (FD; sick according to both PD and HD), and lung lesion diagnosis at slaughter (LLD; lung consolidation ≥ 10% and pleurisy score of 2, or pleurisy score of 3).

Multiple identified metabolites and unknown NMR features (peaks) showed high correlations with BRD status ranging from +0.68 to -0.71 ($P < 0.001$). The strongest correlations were found with PD and FD indicating that the blood metabolome reflected visual signs of sickness as recorded by pen riders. Tyrosine, citrate, glutamine, valine and alanine were lower in BRD case animals compared to controls. Hydroxybutyrate, glucose chains, isoleucine, phenylalanine and creatine were higher in BRD cases compared to control animals ($P < 0.001$). However, the largest differences between case and control animals were found for metabolites which could not be identified.

Blood NMR demonstrated high accuracy (Acc) at detecting BRD defined by PD (Acc=0.85) and FD (Acc=0.81) but were less accurate to detect animals defined as sick according to TD (Acc=0.65), HD (Acc=0.67), LAD (Acc=0.61) and LLD (Acc=0.71). All diagnosis methods required one chemical feature (peak) or metabolite but HD and LLD used 3 and 5 peaks, respectively. The lower accuracy of TD, LAD, HD, and LLD could be due to the metabolome profile reflecting clinical signs at the time when the sample was taken. However, clinical signs such as rectal temperature or lung lesions at slaughter may have peaked or developed either prior to or following blood sample collection.

Future work in this area should incorporate frequent blood sampling and clinical measures starting before exposure to BRD and going throughout infection to slaughter (e.g. serial slaughter of animals). Blood samples taken immediately prior to slaughter could allow searching for biomarkers related to lung lesions. The analyses of the metabolome profile of animals upon entry into the feedlot could also allow the discovery of biomarkers for diagnosis at this critical point in time. Additionally, independent datasets from different feedlots should be collected to evaluate the diagnostic methods and models developed in the present study. Further experimentation to identify those unknown features in blood relevant for BRD diagnosis discovered in the present study should also be pursued with the aim of developing simple crush-side tests.

The results indicate that one to five metabolites in the blood of feedlot cattle are useful indicators to detect or confirm BRD in feedlots however the chemical structure of some of these need to be determined. Blood metabolomics shows great potential to aid in defining and confirming BRD cases under commercial feedlot conditions. This technology could help reducing the use of antimicrobial treatments and economic losses, and improve the effectiveness of treatment protocols and productivity.

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1 Background

Bovine Respiratory Disease (BRD) is a multifactorial disease complex of high significance to the Australian feedlot industry. Bovine Respiratory Disease results from a combination of environmental and physiological stressors prior to and upon feedlot entry such as transportation, mixing of unfamiliar animals and subsequent exposure to viral and bacterial agents (Cusack, McMeniman & Lean, 2003). Approximately 60 to 70% of the morbidity and mortality that occurs in Australian feedlots has been attributed to BRD (Sackett et al., 2006). The complex nature of BRD makes establishing a universal 'gold standard' for BRD case definition problematic (Fulton & Confer, 2012). Common diagnosis methods in feedlots currently rely on clinical signs of illness detected by pen riders, usually combined with rectal temperature or lung auscultation, or both, to trigger antimicrobial treatment protocols (Wolfger et al., 2015b). These diagnosis methods have been shown to have varying accuracy in diagnosing BRD, therefore exploration of alternative diagnosis methods is warranted (Buczinski et al., 2014; Mang et al., 2015; White & Renter, 2009).

Metabolomics is an emerging field of science that monitors alterations in small metabolite cell function (Goldansaz et al., 2017). Small metabolites within circulation in the body include lipids, amino acids, vitamins and sugars. These metabolite biomarkers can provide an insight into the response of an animal's biological systems to disease and can therefore be used to indicate the presence of disease, and classify animals into groups using statistical techniques for predictive modelling (Moore et al., 2007; Xia et al., 2013). Biomarkers are now routinely used in humans to screen for over 30 different disorders including diabetes and heart disease, where metabolic profiling has demonstrated a high accuracy for disease detection (Slupsky et al., 2009; Wang et al., 2011). Metabolomics has also shown potential to improve diagnosis of pneumonia in humans, with numerous metabolite biomarkers identified related to the host response to infection (Laiakis et al., 2010; Seymour et al., 2013; Stringer et al., 2011). More recently metabolomics has been used to predict and identify production traits in cattle such as residual feed intake and reproductive performance, as well as in detecting metabolic and reproductive disorders such as ketosis and metritis (Enjalbert et al., 2001; Karisa et al., 2014; Ospina et al., 2010).

The research to date has shown promising preliminary results in relation to identifying biomarkers for metabolic and reproductive diseases in cattle, however little work has been done on infectious diseases such as BRD. One recent study used NMR-based metabolomics to identify twelve statistically significant metabolites in calves with bronchopneumonia, indicating the potential of metabolomics to diagnose BRD affected animals (Basoglu et al., 2016). As with most studies concerned with biomarker discovery for disease diagnosis in cattle (De Buck et al., 2014; Enjalbert et al., 2001; Goldansaz et al., 2017), this study lacked the sample size and validation necessary to ensure reproducibility. There has also been little work done on characterizing the metabolome of healthy control animals to identify baseline values (Goldansaz et al., 2017). The current study attempted to address these gaps in the previous literature using a larger sample size, case and control animals, and training and validation datasets under commercial conditions to ensure reproducibility of the detection methods on future datasets.

The objective of the current study was to use proton nuclear magnetic resonance (H1 NMR) of the blood metabolome to predict and classify BRD cases in feedlot cattle. We hypothesized that the blood metabolome could be used to classify animals into sick and healthy against the most commonly used reference diagnosis methods and could therefore have the potential to be used as a diagnostic tool for BRD in the future.

2 Project objectives

2.1.1 Complete metabolomics analysis of blood samples via 1H NMR and LC/MS

2.1.2 Examine relationships between the metabolome and visual determination of BRD (sick or healthy), cumulative BRD score, nasal swab status, seroconversion and serological increase to BRD causative viruses, rectal temperature, whisper score and lung abscess score for feedlot animals.

3 Methodology

This experiment was reviewed and approved by the Institutional Animal Ethics Committee, Research Integrity and Ethics Administration, The University of Sydney, Australia (Approval # 2016/1118).

3.1 Animals and management

This experiment was conducted at a commercial cattle feedlot in Southern NSW, Australia. Four pens of mixed-breed steers (total n = 898; 300, 266, 91 and 241 steers in pen 1, 2, 3 and 4, respectively) were followed from induction to slaughter. Animals were slaughtered between 112 and 117 days on feed (DOF). Animals were sourced from multiple locations and were either purchased through saleyards (n=788) or direct consignment from farms (n=110). The breeds used in the study were Angus (n=187), Angus crosses (Hereford x Angus; n=156), Bos indicus crosses (n=29), British crosses (British breed mix and less than 75% Angus; n=82), European (Simmental, Charolaise or Limousin; n=123), Hereford (n=226), Murray Grey (n=59) and Shorthorn (n=36). Cattle were inducted at approximately 1 to 2 years of age (0 to 2 permanent teeth) and weighed 432 ± 51 kg at the start of the trial (mean ± standard deviation). Induction was staggered so that the first pen was inducted on the 28th February 2017, the second and third pens were inducted on the 8th March 2017 and the fourth pen was inducted on the 15th March 2017. At induction into the feedlot, all cattle had initial body weight recorded and were administered the standard feedlot induction treatments which included hormonal implant (Revalor S; Coopers Animal Health, NSW, Australia), and vaccination against Mannheimia haemolytica (Bovilis MH, Coopers Animal Health, NSW, Australia), bovine herpes virus-1 (BHV-1; Rhinogard, Zoetis Animal Health, New Jersey, USA), clostridial diseases (Tasvax 5 in 1, Coopers Animal Health, NSW, Australia) and anti-parasitic injection (Bomectin, Bayer, Leverkusen, Germany). Following induction, animals were sent to production pens and were transitioned through a starter diet, and two intermediate diets before being placed on the finisher diet at 18 DOF. Feedlot diets were based on steam-flaked barley.

3.2 Clinical measurements and sampling

Animals were checked daily by trained feedlot pen riders for visual signs of BRD and scored using a modified version of the Wisconsin calf scoring chart (McGuirk, 2008). The adapted scoring system included seven visual symptoms: lethargy, head carriage, laboured breathing, cough, nasal discharge, ocular discharge and rumen fill to assess BRD presence (Mcmeniman & Batterham, 2016). Animals were assigned a score between 0 and 3 for each of these seven visual symptoms. Animals with visual signs of BRD (n=148; a score of greater than 0 on the visual score chart) were pulled from their pens and taken to the hospital shed for further inspection and clinical data collection. An equivalent number of visually healthy animals (n=152) with a score of 0 on the visual score chart

were pulled from the same pen each day and taken to the hospital shed along with the visually sick animals. Animals were between 2 and 42 DOF at the time of sampling when developed clinical signs of BRD for the case animals.

At the hospital chute, time, date, visual ID, RFID, pen, sex and body weight were recorded for both visually sick and visually healthy (control) animals. Rectal temperature was collected using a GLA M750 thermometer (GLA Agricultural Electronics, CA, USA) fitted with 10 cm probe and inserted for 8 to 15 seconds until peak temperature was reached. Lung auscultation score was recorded using a Whisper Computer Assisted Lung Auscultation system (Geissler Corporation, MN, USA). The diaphragm of an electronic stethoscope was held over the 5th intercostal space of the right thoracic wall, approximately 10 cm posterior to the elbow and lung sounds recorded for 8 seconds. Recorded lung sounds were then transmitted wirelessly to a computer containing software to analyse the lung sounds. The Whisper program classifies lung sounds into scores from 1 to 5 (1 = normal, 2 = mild acute, 3 = moderate acute, 4 = severe acute, 5 = chronic). Blood samples for metabolomics analyses were collected from the tail vein of each case and control animal at pulling in a 10 ml Lithium Heparin BD Vacutainer. Samples were placed on ice until they could be centrifuged (2500 *g* for 20 min) within an hour of collection. The plasma was then transferred to 1.5 ml Eppendorf safe-lock micro test tubes and frozen at -20 ºC until sampling was completed, and then sent to the laboratory for storage at -80 ºC until analysis.

All animals were followed through to slaughter and lung abnormalities recorded using the lung scoring method explained by Theurer et al. (2013), where the consolidation on each lobe was summed to form a total percentage of lung consolidation. Pleurisy was recorded using a scoring system of 0 to 3 developed by Dr Kev Sullivan, Bell Veterinary Services (Table 1). Carcass weight was recorded on the processing chain for all trial animals.

Table 1. Pleurisy scoring system used at slaughter to define lesions associated with Bovine Respiratory Disease.

Pleurisy Score	Description
0	No pleurisy or pleuritic tags evident on the lungs
1	Tags between lobes or small pleuritic tags on the lung surface
2	Significant pleuritic tags on the lung surface or small pieces of lung adhered to the thoracic wall or significant tags on the lung margins (fringing) or between lobes that could not be broken apart by the inspector
3	All the lung adhered to the thoracic wall with no lung present on the offal table for scoring

3.3 Diagnosis of Bovine Respiratory Disease

In order to evaluate the utility of the blood metabolome in predicting BRD, six commonly used methods of BRD diagnosis in feedlots were used as reference methods as outlined below. The pen rider visual diagnosis definition (PD) classified animals as BRD cases if the BRD visual scores previously recorded were greater than zero whereas control animals had a score of zero. For the rectal temperature (TD) definition, animals with a rectal temperature greater than or equal to 40^{o}C were considered cases and animals with a rectal temperature less than 40^{o}C were considered controls (Schaefer et al., 2012; Wolfger et al., 2015a). For the lung auscultation diagnosis (LAD) definition, animals with a Whisper score of 2 or greater were considered cases and animals with a score of 1 were considered controls (Mang et al., 2015). For the hospital diagnosis (HD) definition, BRD cases were any animals with rectal temperature ≥ 40 ºC or Whisper lung auscultation score ≥ 2, or both. Pen rider and hospital diagnosis were combined to form a feedlot diagnosis (FD) definition as routinely used to trigger veterinary treatment protocols in feedlots where a visually sick animal is pulled from its pen and taken to the hospital shed to confirm presence of BRD by measuring rectal temperature and lung auscultation. According to the FD definition, an animal was classified as a BRD case if it showed visual signs of BRD (visual score > 0) and rectal temperature ≥ 40 ºC, or showed visual signs of BRD and had Whisper score ≥ 2. Thus, a BRD case animal according to FD was also a case for both the PD and HD concurrently. In contrast, a control animal according to FD was an animal that was a control according to either PD or HD. The lung lesions diagnosis (LLD) definition used in this study incorporated both the percentage of lung consolidation and pleurisy score at slaughter. According to the LLD definition, an animal was classified as a BRD case if it had either pleurisy score of 3 (regardless of lung consolidation percentage), or pleurisy score 2 with lung consolidation of 10% or more. An animal was classified as a LLD control if it had either a pleurisy score 1 or lung consolidation of less than 10%, or both.

Animals in the present study received the first BRD treatment at the hospital shed with either Tulathromycin (Draxxin, Zoetis Animal Health, New Jersey, USA) for animals with lung auscultation score of 2 and rectal temperature ≥ 40 ˚C, or lung auscultation score 3 regardless of rectal temperature; or Tilmicosin (Tilan, Elanco Animal Health, West Ryde, NSW, Australia) for animals with lung auscultation score of 2 and rectal temperature less than 40 ˚C, or rectal temperature ≥ 40 ˚C

and lung auscultation score of 1. Blood sampling occurred only in animals treated for their first time for BRD or controls that were not treated for BRD previously since arrival to the feedlot. Animals with rectal temperature < 40 ºC and lung auscultation score of 1 were not treated regardless of visual signs.

3.4 Metabolomics sample analysis

Plasma samples were thawed, centrifuged at 12,000 g for 5 min at 4 °C and 350 µl aliquots were transferred to 5 mm NMR tubes where 350 µl of plasma buffer were added (Dona et al., 2014; Dona et al., 2016). Quality control samples were obtained by mixing the thawed plasma of several animals from the same cohort and prepped in the same way as the samples. Samples were placed in a rack and one control was run every 20 samples. Samples and controls were analysed with a Bruker Avance III 600 MHz spectrometer equipped with a 5-mm TCI cryoprobe (Bruker, MA, USA). Samples were run under automation mode using a SampleJet with all samples refrigerated at 278 K until just prior to acquisition. Data was collected at 310 K for a total of 20 minutes. Two water suppressed 1D 1H NMR spectra were acquired using the noesygrrp1d and cpmgpr1d pulse sequences (32 scans collected for each experiment). Irradiation of the solvent (water) resonance was applied during pre-saturation delay (4.0 s) for all spectra and the noesy during the mixing time (0.01 s). The pulse sequence parameters including the 90° pulse (~ 12 µs) receiver gain (~ 100) were optimized for each sample set run. The data were collected with approximately 96 k (noesy) or 32 k (cpmg) real data points and processed with an exponential line broadening of 0.3 Hz prior to Fourier transformation. Raw spectrums were imported into Matlab (MathWorks, Natick, MA), automatically phased, baseline corrected and referenced to the α-C1H glucose doublet occurring at 5.23 ppm (Dona et al., 2016). The water peak was truncated to reduce analytical variation. Probabilistic quotient normalization of the spectrums was performed across all samples (Dona et al., 2016). The normalized spectrums were then analysed using Principal Component Analysis which determined that the quality controls clustered together when plotted (Dona et al., 2016). Once aligned and normalized, the spectrums were processed using Standard Recoupling of Variables to calculate the start and end points of each peak or feature (Craig et al., 2006). Then, the area under each peak was calculated which represents the relative abundance of each peak (Dona et al., 2016). Raw spectrums were then imported into Chenomx NMR Suite (Chenomx, Edmonton, Canada) to identify metabolites using reference libraries (Dona et al., 2016).

3.5 Data processing and statistical analysis

Data were analyzed using the statistical software package SAS (v 9.4, SAS Institute, NC, USA). Pearson correlation coefficients were calculated between the relative concentration of a metabolite or unknown peak and clinical measures (e.g. rectal temperature) or the correlation with diagnosis methods after assigning a value of one to BRD cases and zero to the controls. Mixed-effects linear regression models were used to compare case and control groups for induction weight, live weight at first pull, rectal temperature, lung auscultation score, lung consolidation percentage, pleurisy score, carcass weight and average daily gain (ADG) to pull for the case and control groups for all six reference diagnosis methods outlined previously (PD, HD, FD, TD, LAD and LLD). Average daily gain to pull was calculated as the difference between induction weight and hospital weight at pulling divided by DOF at pull. Fixed effects included in the models were diagnosis method, induction weight as a covariate (for pull weight, ADG to pull and carcass weight) and breed. DoF and vendor (or source) were also included as fixed effects for pull weight, rectal temperature, lung auscultation score and ADG to pull. Pen was a random effect for this analysis. The models were constructed by including all potential effects previously mentioned and removing nonsignificant ($P > 0.05$) effects

one at a time in a backwards manner so the final model included only variables with values of $P \leq 0.05$. Data for lung consolidation was log transformed for skewness prior to analysis. Animals that had metabolomics samples taken initially and were then pulled for BRD after the blood sampling period ended were removed from all analysis relating to slaughter data (e.g. carcass weight and lung consolidation) because this data would not be representative of the blood sample.

Classification and regression trees (Breiman et al., 1984) were used to develop models to predict BRD status using the blood metabolome and identify potential biomarkers for the six BRD diagnosis definitions. All peaks identified by the NMR were used to develop the prediction models however it is important to point out that a metabolite may be comprised by one or more 'peaks' or features (Goldansaz et al., 2017). Models were developed using entropy to grow the trees (JinFu, 2010) with cost-complexity pruning (Breiman et al., 1984). The data was partitioned into training (model development; n=150) and validation data sets (n=150) before analysis. All the metabolite peaks were added to the model as predictors with diagnosis method as the dependent variable to be predicted. Sensitivity, specificity and accuracy (100 minus error rate) was calculated using the number of true positive, true negative, false positive and false negative. Sensitivity was defined as the frequency with which the model correctly identified BRD cases according to each reference diagnosis method. Specificity was defined as the frequency with which the model correctly identified controls according to each reference diagnosis method. Accuracy was calculated as the sum of the proportion of true positive and true negative animals. Sensitivity and 1- specificity were plotted against each other to produce an area under the curve (AUC) which determined the overall accuracy of the models. Parametric receiver operating characteristic (ROC) curves were created for the final models to determine their overall accuracy at predicting BRD using the blood metabolome.

4 Results

The number of animals classified as cases and controls for each of the six diagnosis methods are displayed in Table 2. Following initial pulling based on visual observation by the pen riders, 49.3% of all 300 animals pulled by the pen riders were considered cases according to PD. However, less than one third (31%) of all pulled animals exhibited rectal temperatures ≥ 40 ºC and were therefore considered BRD cases according to TD. In contrast, the LAD definition classified 62.3% of all pulled animals as cases and the HD definition classified 66.7% of all pulled animals as cases due to high rectal temperature or high lung auscultation score, or both. Only 24% of all animals were classified as BRD cases according to LLD as a result of showing lesions in the lungs or pleurisy at slaughter (Table 2).

When assessing differences between case and control animals, breed was found to have a significant effect on induction weight, lung auscultation score and carcass weight ($P < 0.05$; data not shown). Induction weight as a covariate was found to have a significant effect on hospital pull weight and carcass weight ($P < 0.05$; data not shown), whereas DoF had a significant effect on hospital pull weight, rectal temperature and ADG to pull ($P < 0.05$; data not shown). Induction weight was lower for BRD case animals for PD, HD, FD and TD diagnosis methods ($P < 0.05$) however it was not different between case and control animals for LAD and LLD ($P > 0.05$; Table 3). Weight and ADG at pulling were higher in the control animals compared to the BRD case animals for all diagnosis methods ($P < 0.05$). Rectal temperature and lung auscultation score were higher for case animals compared to control animals across all six diagnosis methods ($P <0.05$; Table 3). Lung consolidation percentage was greater in the case animals compared to control animals for PD, FD and LLD ($P < 0.05$), however no differences in lung consolidation existed between BRD case and control animals for HD, TD and LAD ($P > 0.05$). Carcass weight was higher in the controls compared to the case animals for all six diagnosis methods ($P > 0.05$), with the greatest difference between groups for PD and FD methods (Table 3).

Table 2. The number of observations classified as cases or controls for the six reference diagnosis methods to classify feedlot steers as case or control for Bovine Respiratory Disease.

Diagnosis method	Cases		Controls		Total
	Number	Percent	Number	Percent	
Pen Rider	148	49.3	152	50.7	300
Temperature	93	31.0	207	69.0	300
Lung Auscultation	187	62.3	113	37.7	300
Hospital	200	66.7	100	33.3	300
Feedlot	138	46.0	162	54.0	300
Lung Lesions	66	24.3	206	75.7	272

Table 3. Performance and clinical measures for six reference diagnosis methods of Bovine Respiratory Disease case and control groups in feedlot cattle.

	Pen Rider Diagnosis			**Hospital Diagnosis**			**Feedlot Diagnosis**			**Temperature Diagnosis**			**Lung Auscultation Diagnosis**			**Lung Lesion Diagnosis**		
	Case	Control	P-value	Case	Control	P-value	Case	Control	P-value	Case	Control	P-value	Case	Control	P-value	Case	Control	P-value
Induction weight (kg/hd)	426 ± 10.6	442 ± 10.4	0.005	430 ± 10.5	444 ± 10.8	0.03	426 ± 10.6	442 ± 10.4	0.004	424 ± 10.9	440 ± 10.1	0.009	**432 ± 10.5**	**440 ± 10.8**	**0.15**	**452 ± 9.4**	**453 ± 4.8**	**0.90**
Pull weight (kg/hd) [A]	444 ± 5.5	481 ± 5.5	<0.001	454 ± 6.1	480 ± 6.6	<0.001	444 ± 5.9	480 ± 5.8	<0.001	444 ± 6.1	472 ± 5.5	<0.001	455 ± 6.1	475 ± 6.5	<0.001	452 ± 8.1	473 ± 3.3	0.02
ADG to pull (kg/hd/day) [A]	-0.27 ± 0.31	1.80 ± 0.31	<0.001	0.13 ± 0.35	1.63 ± 0.37	<0.001	-0.31 ± 0.31	1.60 ± 0.30	<0.001	-0.32 ± 0.33	1.17 ± 0.29	<0.001	0.13 ± 0.36	1.42 ± 0.37	<0.001	-0.13 ± 0.57	1.03 ± 0.43	0.01
Rectal temperature (°C)	40.0 ± 0.06	39.2 ± 0.06	<0.001	39.8 ± 0.06	39.0 ± 0.08	<0.001	40.0± 0.06	39.2 ± 0.06	<0.001	40.4 ± 0.05	39.1 ± 0.04	<0.001	39.8 ± 0.06	39.2 ± 0.08	<0.001	39.8 ± 0.16	39.5 ± 0.06	0.03
Lung auscultation score	1.97 ± 0.06	1.44 ± 0.05	<0.001	2.03 ± 0.03	1.02 ± 0.04	<0.001	2.02 ± 0.05	1.42 ± 0.05	<0.001	1.95 ± 0.07	1.58 ± 0.05	<0.001	2.11 ± 0.02	1.00 ± 0.03	<0.001	1.87 ± 0.12	1.61 ± 0.05	0.03
Lung consolidation (%)[B]	5.08 ± 0.60	2.60 ± 0.47	<0.001	**3.96 ± 0.52**	**2.74 ± 0.56**	**0.48**	5.14 ± 0.61	2.61 ± 0.47	<0.001	**4.00 ± 0.69**	**3.24 ± 0.46**	**0.08**	**3.80 ± 0.53**	**2.97 ± 0.55**	**0.86**	12.78 ± 0.73	2.23 ± 0.32	<0.001
Pleurisy score	1.58 ± 0.11	1.30 ± 0.11	0.003	**1.31 ± 0.10**	**1.27 ± 0.10**	**0.72**	1.46 ± 0.10	1.22 ± 0.08	0.01	**1.44 ± 0.12**	**1.25 ± 0.09**	**0.07**	**1.29 ± 0.09**	**1.30 ± 0.10**	**0.87**	2.06 ± 0.14	1.19 ± 0.07	<0.001
Carcass weight (kg/hd) [A]	345 ± 3.38	364 ± 2.65	<0.001	354 ± 3.02	362 ± 3.25	0.03	345 ± 3.45	363 ± 2.66	<0.001	349 ± 3.98	360 ± 2.65	0.004	353 ± 3.02	362 ± 3.15	0.02	344 ± 5.77	359 ± 2.56	0.01

[A] Induction weight was used as a covariate.

[B] P-values for lung consolidation were obtained after log transformation of the data.

The NMR spectra resulted in 323 peaks or features after processing the raw spectrums using the standard recoupling of variables (data not shown). However, only 112 out of 323 features were assigned to one of 44 metabolites identified using Chenomx (Fig. 1). Pearson correlation coefficients between the relative concentration of identified metabolites and health status value ranged between +0.64 to -0.66 with the strongest correlations found for PD and FD (Fig. 1). The strongest positive Pearson correlations or increase in the relative concentration of metabolites in sick animals for FD were found for hydroxybutyrate (r = 0.62), glucose chains (r = 0.61), phenylalanine (r = 0.56), and creatinine (r = 0.47; P < 0.001; Fig. 1). The largest negative correlations or lower relative concentration of metabolites in sick animals for FD was found for tyrosine (r = -0.63), glutamine (r = -0.61), citrate (r = -0.60), and valine (r = -0.47; Fig. 1).

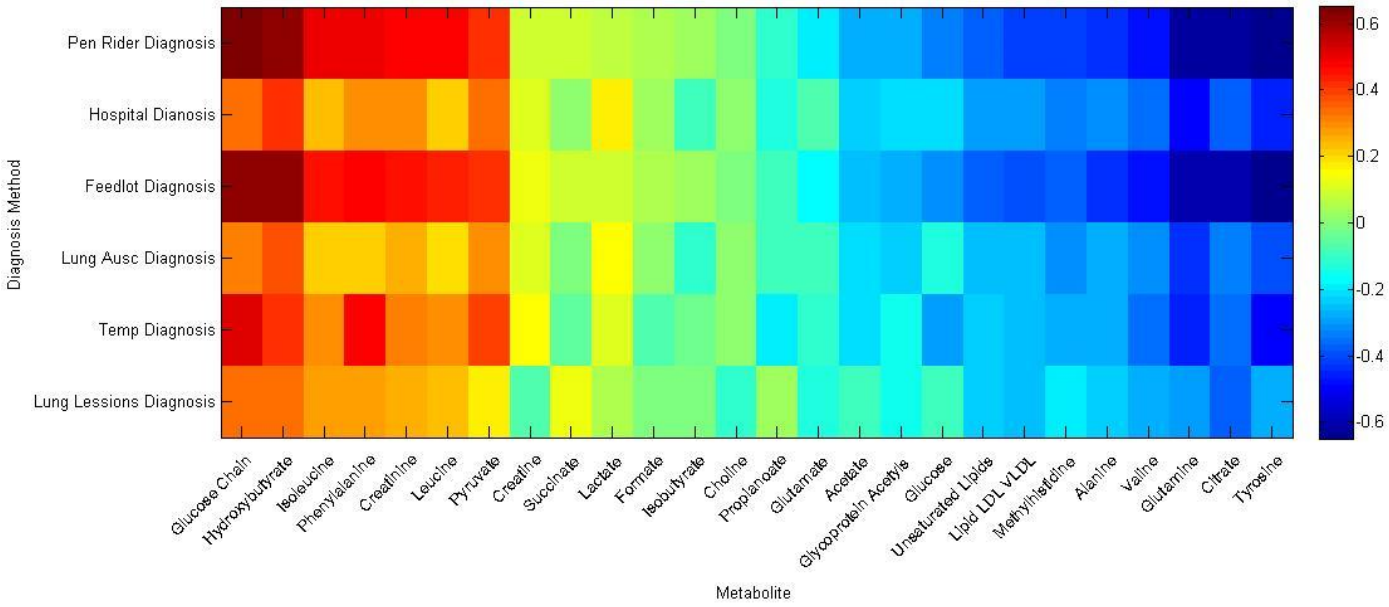

Fig. 1. Heat map showing Pearson correlation coefficients between the relative concentration of plasma metabolites and Bovine Respiratory Disease defined through six reference diagnosis methods based on visual signs (Pen Rider), clinical signs of high rectal temperature (Temp) or lung auscultation scores (Lung Ausc) or either of both (Hospital), concurrent visual and clinical signs (Feedlot Diagnosis), and lung lesions at slaughter (Lung Lesions). LDL: low density lipids; VLDL: very low-density lipids.

However, there were peaks or features that could not be identified or assigned to a chemical structure which yielded slightly stronger correlations with health status ranging from +0.67 to -0.71 (Fig. 2). Furthermore, 71.2% of all 323 peaks showed significant correlations with Pen Rider Diagnosis value ($P < 0.05$; data not shown).

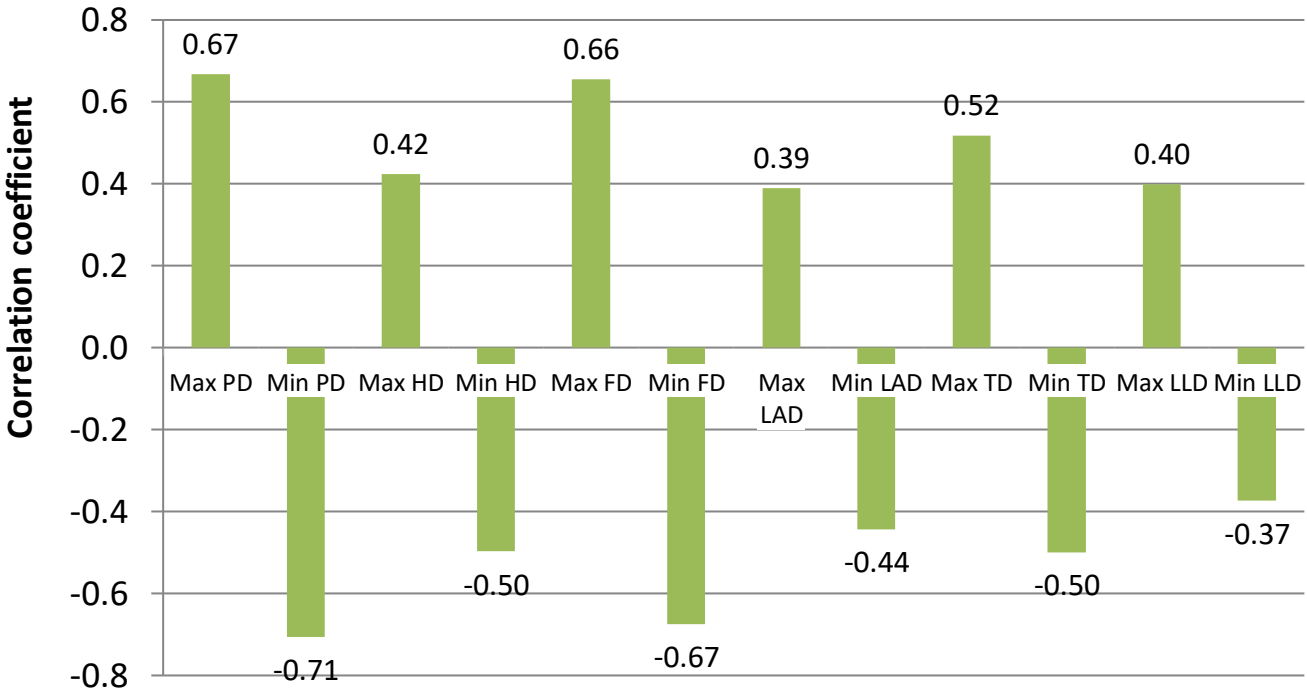

Fig. 2. Maximum and minimum Pearson correlation coefficients between the relative concentration of chemical structures in plasma and the status of Bovine Respiratory Disease in feedlot cattle. BRD was defined through six reference diagnosis methods based on visual signs (PD), high rectal temperature (TD), lung auscultation scores (LAD), either high temperature or lung auscultation (HD), concurrent visual signs, temperature and lung auscultation (FD), and lung lesions at slaughter (LLD). Relative concentration was measured through the area under the peaks or features in the NMR spectra.

The sensitivity, specificity and accuracy of the models developed to classify an animal as a case or control for each diagnosis method are shown in Table 4. Detecting BRD cases from the blood metabolome was generally good for the training datasets, with accuracy decreasing for the validation data sets for all reference diagnosis methods. However, the reduction in the accuracy with the validation dataset compared to the training dataset was smaller for some diagnosis methods such as PD (-2%) compared to others such as LLD (-19%; Table 4). The metabolome profile best predicted BRD cases defined by the pen rider visual diagnosis definition as it showed the highest accuracy (Acc = 85%) in the validation data set. This accuracy was achieved with only two leaves and one peak or feature in the classification tree of the final pruned tree. Interestingly, Peak 92 showed the strongest negative correlation with pen rider diagnosis value (R^2=0.70; data not shown). A metabolite search did not allow identification of the chemical compound although it is a singlet occurring at 5.39 ppm (data not shown).

Table 4. Sensitivity and specificity of H NMR metabolomics to detect Bovine Respiratory Disease in feedlot cattle defined with six reference diagnosis methods: pen rider diagnosis (PD), hospital diagnosis (HD), feedlot diagnosis (FD), rectal temperature diagnosis (TD), lung auscultation diagnosis (LAD) and lung lesion diagnosis (LLD).

Diagnosis method	Dataset	Sensitivity	Specificity	Accuracy	AUC [A]	N leaves [B]	N peaks [C]	Peak Number Metabolite ID
Pen Rider	Training	0.81	0.93	0.87	0.87	2	1	92
	Validation	0.82	0.87	0.85	0.85	2	1	Unknown (Singlet at 5.39 ppm)
Hospital	Training	0.77	0.85	0.79	0.81	2	1	227
	Validation	0.79	0.54	0.70	0.67	2	1	3-Hydroxybutyrate
Feedlot	Training	0.99	0.88	0.93	0.94	4	3	55, 211, 158
	Validation	0.88	0.74	0.81	0.83	4	3	Tyrosine, Citrate, 3-hydroxybutyrate
Temperature	Training	0.76	0.88	0.85	0.82	2	1	34
	Validation	0.52	0.77	0.69	0.65	2	1	Phenylalanine
Lung Auscultation	Training	0.80	0.73	0.77	0.76	2	1	123
	Validation	0.77	0.45	0.64	0.61	2	1	Lactate
Lung Lesions	Training	0.76	0.97	0.92	0.90	6	5	219, 130, 292, 305, 25
	Validation	0.38	0.89	0.74	0.71	6	5	Citrate, Unknown, Unknown, Leucine, Unknown

[A] AUC = Area Under the Curve

[B] N leaves = number of leaves in final tree

[C] N peaks = number of peaks selected in final tree by the classification tree analysis

The feedlot diagnosis definition produced a model with the highest accuracy in the training dataset but a slightly lower accuracy in the validation data set which had 19% of the observations misclassified and an AUC of 0.83 (Table 4). This model used 3 peaks to classify an animal as sick or healthy, with peak 55 being the most important and belonging to one of two peaks of tyrosine (data not shown). The other 2 peaks selected to predict FD were identified as citrate (peak 211) and 3-hydroxybutyrate (peak 158). Detecting a sick animal as defined by LAD showed the lowest accuracy (Acc = 0.64), with 36% of animals being misclassified in the validation dataset and an AUC of 0.61. Using the blood metabolome to detect a BRD case as defined by LLD yielded high accuracy and AUC for the training dataset however the sensitivity was only 38% when the prediction model was applied to the validation dataset (Table 4). Detecting BRD cases as defined by LLD used 5 peaks with 3 of them not identified (unknown) and the other 2 being citrate and leucine.

5 Discussion

The present study was carried out in a commercial feedlot setting utilizing steers of mixed breeds and sourced from both saleyards and private vendors to ensure robustness and generalization of the detection methods under investigation. A case-control study was selected to capture both BRD affected and healthy animals for appropriate model development and evaluation. The major limitation of research evaluating BRD detection and diagnosis is the lack of a gold standard to define a truly BRD-affected animal (Wolfger et al., 2015). This was the reason to use six reference diagnosis methods instead of trying to define a gold standard. The present study also analysed blood samples taken at induction and pulling to assess seroconversion to BRD causative viruses, and nasal swabs taken at pulling to assess viral shedding using qPCR (BHV-1, BVDV, BRSV, BPI3 and BoCV; data not shown). These data were found to provide limited utility to help differentiating sick and healthy animals because most animals were either pre-exposed to all viruses or seroconverted to at least one of the viruses because of the vaccination program at induction (data not shown). In addition, only 18.1% of all animals or 29.0% of the visually sick animals were shedding a virus at the time of pulling and sampling. These data were therefore not considered for further analysis or to diagnose BRD. The sample size for lung lesions at slaughter (LLD) was smaller than for the other reference methods due to both mortalities during the trial (n = 19) and exclusion from analysis of animals that were pulled as BRD cases after blood samples were taken for metabolomics. These latter animals were not included for the analysis of lung lesions because the blood sample may has been taken before the BRD event causing histopathological lesions of the lungs observed at slaughter have occurred (i.e. pulled as controls for blood sampling and as BRD case after blood sampling was finished).

In the absence of a true gold standard, the ability of blood metabolomics to detect BRD cases was determined for six reference diagnosis methods commonly used in the industry. Case animals were initially pulled due to visual signs of BRD, and a visually healthy animal was pulled for every case animal. Visual signs included nasal and ocular discharge, breathing difficulty and lethargy. This approach resulted in a balanced number of case and control animals for PD. However, over two thirds of all animals pulled by the pen riders were classified as HD cases upon further examination at the hospital shed particularly due to high lung auscultation scores. The proportion of animals with high rectal temperature was only 31% however these also contributed to the increased number of HD cases from 187 in LAD to 200 in HD. This resulted in 41.5% of the visually healthy animals pulled by pen riders receiving antimicrobial treatment. This disparity between visual signs and other clinical measures such as elevated temperature and abnormal lung sounds has been well reported in the literature and are believed to be due to the prey instinct of cattle which causes them to hide signs of illness (Portillo, 2014; White & Renter, 2009). However, this hypothesis suggests that visual signs are not a good indicator of BRD and that rectal temperature and lung auscultation are more suitable

indicators. This contrasts with the findings of the present study where the strongest correlation between the blood metabolome and any clinical measure was found for visual signs of BRD, i.e. PD and FD.

Animal performance and clinical results were included in the present study to illustrate differences between case and control groups for the different reference diagnosis methods. BRD cases had lower induction weight than controls, which affected final carcass weight despite the fact induction weight was used as a covariate to correct for their lower induction weights. Several studies have suggested that lighter animals are at greater risk of BRD than heavier animals which is supported by our results (Martin et al., 1989; Sanderson, Dargatz & Wagner, 2008). In general, BRD case animals demonstrated lower performance across all six diagnosis methods. However, the largest differences in performance between case and control animals (BW at pulling, ADG from induction to pulling, and carcass weight) were observed for PD and FD. For instance, the difference between case and control animals for ADG to pull (time of blood sampling) was largest for PD (2.07kg/d) and FD (1.91 kg/d) and smallest for LLD (1.16 kg/d). In contrast, the lowest differences in carcass weight between case and control animals were demonstrated for HD and LAD whereas PD and FD still showed the largest difference between groups. Interestingly, PD and FD were the only diagnosis methods apart from LLD that showed a significant difference between case and control animals for pleurisy score and lung consolidation percentage. These results on animal performance and clinical data suggest that using the visual signs as done by pen riders in the present study may be the most accurate method to detect BRD affected animals. However, a low proportion of the PD and FD cases showed lung lesions at slaughter suggesting that the infection may have resolved leaving no lesions. The strongest correlations between disease status and blood metabolomics were also reported for PD and FD supporting this hypothesis. However, caution should be taken when interpreting these results because the present study was not designed to confirm the best method for evaluating the impact of BRD and the discovery of a gold standard is still needed. The results also highlight the need to consider temporal changes of the measured variables from prior to exposure and the onset of disease to adequately evaluate detection methods. In conclusion, PD and FD displayed the greatest utility in differentiating between BRD affected and healthy animals from both the live animal and slaughter data. In contrast, HD, TD and LAD appeared to have the least utility in differentiating between case and control animals based on clinical examination and performance data.

The NMR spectra obtained in the current study identified 323 peaks or features however multiple peaks or features could belong to the same metabolite (Goldansaz et al., 2017). Twelve out of the 323 peaks were selected by the classification tree models. Interestingly, none of the peaks of importance selected were the same for the six reference diagnosis methods, which may indicate that the underlying metabolic mechanisms involved in, or affected by BRD, may differ depending on the clinical measures used to define BRD. Alternatively, this may indicate that different clinical signs reflected through the blood metabolome through different metabolites. Previous studies analysing metabolites of BRD-affected cattle have identified and quantified up to 30 metabolites (Aich et al., 2009; Basoglu et al., 2016). Out of the 30 metabolites identified by Basoglu et al. (2016), seven were found to be significantly different between healthy and diseased dairy calves defined through visual scoring. These were propionate, 2-methylglutarate, ethanol, acetate, dimethyl-sulfone, allantoin and phenylalanine. These metabolites, except phenylalanine, differ from those found to be important to define BRD cases in the present study which could be due to differences in analytical and statistical techniques used, the type of animals and possible causative agents. Metabolites with negative correlations with disease in the present study indicate that the relative concentration of those metabolites was lower in sick animals compared to healthy animals. In agreement with the present study, phenylalanine also increased in sick animals of Basoglu et al. (2017) however the 4 metabolites with strongest correlation with disease in the present study were not significant in the study cited although tyrosine and hydroxybutyrate showed similar direction of the response. Findings from the present study agree with the lower relative concentration in sick animals of tyrosine, citrate and glutamate at 12 hours after artificially infecting beef steers, and to the increase

in phenylalanine and leucine reported by Terril et al. (2010). The rest of the metabolites with strongest correlations with disease of the present study differed between the two studies. However, it is important to note that the metabolic changes observed by Terril et al. (2010) were inconsistent and partly dependent on the type of infection (viral or bacterial) and time after infection. An interesting difference between the present study and Basoglu et al. (2016) is the fact that in the present study a larger number of metabolites and peaks seemed to be significantly correlated to disease status. For example, 71.2% of all 323 peaks showed significant correlations ($P < 0.05$) with Pen Rider Diagnosis values. Another study assessing biomarkers for BRD prediction found that lactate and cortisol predicted mortality and conversely, higher glucose concentrations were associated with survival (Aich et al., 2009). The present study has identified 44 metabolites from the 323 peaks with a total of 12 peaks deemed of importance in differentiating sick from healthy animals across all diagnosis methods. However, the largest differences on the relative concentration of blood components between case and control animals were found for metabolites which could not be identified to assign a metabolite name. For example, peak 92 occurring at 5.39 ppm was the only peak selected by the classification trees to detect BRD cases according to PD however its chemical structure could not be identified. Further research is required to identify this metabolite and its chemical composition. In contrast to PD, FD cases could be detected with high accuracy based on the relative concentration of tyrosine, citrate and 3-hydroxybutyrate. Citrate was negatively correlated with both PD and FD indicating that sick animals had lower relative concentration compared to controls, whereas 3-hydroxybutyrate showed a positive correlation with PD and FD indicating that BRD cases had higher concentration. Citrate is a member of the tricarboxylic acid cycle whereas hydroxybutyrate is one of the ketone bodies that normally accumulate in the bloodstream of cattle under negative energy balance. Interestingly, Baticz et al. (2002) demonstrated that these two compounds follow opposite trends and have a negative correlation in dairy cows after calving. Therefore, the identification of peak 92, and the use of citrate and 3-hydroxybutyrate could assist to objectively define BRD cases in feedlots.

The present study used classification and regression trees to search for biomarkers of disease and to develop prediction models that could be applicable to new datasets in the future. This technique searches for variables (NMR peaks or metabolites) that partition the dataset with the highest accuracy or lowest error rate when classifying an animal as sick or healthy. Classification and regression trees were selected because these allow predictive modelling through the identification and selection of specific biomarkers and are simple to understand. Simple handheld devices based on these biomarkers identified could be implemented as crush-side diagnostic tools for BRD. Other machine learning techniques such as random forests, orthogonal partial least square discriminant analysis, and support vector machines could result in higher accuracy, but these are more complex for practical applications as they may require entire NMR spectrums and consistent peak definitions for predictions in future datasets. It is important to point out however, that the training and validation datasets were not completely independent with the animals being part of the same trial and feedlot. Future work should validate the prediction models developed in the present study with completely independent datasets from different feedlots (Liland, 2011). The robustness of the results is however supported by the fact that the study was performed under commercial conditions, with animals from different sources, breeds, ages, nutritional status, days on feed, diets, and body condition, and sampled on different dates with different environmental conditions.

The sensitivity, specificity and accuracy of the models developed in the present study to predict BRD using the PD and FD definitions are higher than many of the current methods of BRD diagnosis. Methods using white blood cell counts (WBC) have been shown to have sensitivities and specificities ranging from 25 to 78% and 77 to 94%, respectively (Schaefer et al., 2012; Schaefer et al., 2007). Such large ranges in diagnostic accuracy and relatively low sensitivity make WBC of limited value to confirm BRD in feedlots. Acute phase proteins (APPs) have demonstrated a relatively high sensitivity (93%) and specificity (86-93%) to detect BRD with only one to two APPs used so far for laboratory confirmation (Idoate et al., 2015). However, it is important to note that APPs are not specific to

infection but just indicate tissue trauma or inflammation which could be due to several reasons including social stress (González et al., 2008). Stress related hormones such as cortisol have been shown to have high sensitivity (75 to 100%) in detecting BRD however these are not specific to infection or inflammation resulting in low specificity ranging from 52.9 to 53.8% (Schaefer et al., 2012; Schaefer et al., 2007). Other diagnostic methods including behavioural monitoring and infrared thermography have displayed varying accuracy at detecting BRD, with sensitivities and specificities ranging from 60 to 81% and 100 to 87.2%, respectively (Schaefer et al., 2012). In contrast to the present study, Schaefer et al. (2012) reported that both rectal and orbital IR temperature had a sensitivity of 100% and specificity of 97.4% to detect BRD. It is apparent from these results that there is a need for alternative, more accurate diagnosis methods to be developed, and the accuracy of the models developed in the current study indicate the potential of blood metabolomics to classify animals with BRD.

The blood metabolome best predicted visual signs of BRD (PD and FD) and this could be due to the fact that samples were taken on the day when clinical symptoms were most apparent. Therefore, the metabolites identified in the present study could be used as an accurate and objective method of confirming BRD initially detected through subjective clinical observation by the pen riders. Basoglu et al. (2016) reported better accuracy (0.95) compared to the present study however the study had only ten healthy controls and 50 cases, which does not permit adequate comparison, and did not use a validation dataset to test the model performance. Principal Component Analysis (PCA) was used to develop the classification models and it is uncertain how these models would perform on future datasets using a selection of metabolites.

The blood metabolome in the present study had the lowest accuracy in predicting BRD when using TD, LAD, HD and LLD definitions. The low accuracy of the TD, LAD and HD models could likely be due to the fact that only one blood sample was taken at the time the animal exhibited visual signs. However, clinical symptoms (rectal temperature and lung sounds) may not have peaked or been most evident at this time depending on the stage of infection at the time of sampling (Timsit et al., 2011). Daily measurements prior to and after exposure to infection would be required to determine changes in the blood metabolome in relation to visual signs, rectal temperature and lung auscultation. Similarly, the fact that the metabolome was not accurate at classifying BRD according to LLD is not unexpected given the metabolome is highly dynamic and susceptible to rapid change, meaning that the blood composition at the time of sampling is only reflective of that time point (Goldansaz et al., 2017). Lesions evident at slaughter could have developed prior to feedlot entry, or before or after a blood sample was obtained, or alternately lesions could have resolved prior to slaughter. Previous research has reported lesions at slaughter in as few as 37% of animals clinically diagnosed with BRD (Gardner et al., 1999). This latter study also reported that the number of animals that displayed lesions at slaughter and not treated for BRD (29%) was similar to the number of animals with lesions that were treated for BRD (37%). This suggests that lung pathology at slaughter is not always representative of the clinical diagnoses of BRD in the live animal. In addition, lung lesions cannot be used as a diagnosis method of BRD to trigger antimicrobial treatments as it requires slaughter or ultrasonography which is time consuming and requires expertise. It is therefore suggested that lung pathology is an imperfect reference test for the present study as it cannot be correlated to the metabolome profile of the animal at the time the blood sample was taken.

6 Conclusions/recommendations

6.1 Conclusions

The blood metabolome is a useful tool to classify BRD case and healthy animals and could be used as an accurate diagnostic tool in feedlots. Defining BRD cases from visual signs obtained by pen riders

and confirmed with rectal temperature and lung auscultation seems the most accurate diagnosis method. Single measurements of rectal temperature or lung auscultation at pulling, and lung lesions at slaughter may be less accurate to define BRD cases compared to visual signs. At the time of pulling, the metabolome is most closely correlated to visual signs than to rectal temperature, lung auscultation score, blood serology, pathogen shedding from nasal swabs and lung lesions at slaughter. However, it is unknown if the blood metabolome could be able to detect changes in rectal temperature, lung auscultation and lung lesions when these clinical and pathological measures are developing or at their peak. An important finding is that one to five metabolites could be able to accurately detect or confirm BRD in feedlot cattle opening opportunities to develop simple, rapid and practical crush-side tests.

6.2 Recommendations

Future work in this area should incorporate frequent blood sampling and serial slaughter of animals starting before infection with BRD, and blood samples taken immediately preceding slaughter to search for biomarkers related to lung lesions at slaughter. The analyses of the metabolome profile of animals upon entry into the feedlot could also provide more detailed information on the usefulness of this method as a diagnosis tool at this critical point in time. Additionally, independent datasets from different feedlots should be used to evaluate the models developed in the present study. Further research to identify the chemical structure of those blood 'features' which showed high accuracy to define BRD cases is also recommended.

7 Key messages

- Blood biomarkers of BRD in feedlot cattle are a promising tool for the detection and confirmation of the disease with potential to become a crush side diagnostic tool.
- The largest difference between case and control groups in weight at pulling, growth rate to pulling, and carcass weight was found when animals were classified as BRD cases using visual signs only, or visual signs confirmed by high rectal temperature and lung auscultation scores at pulling.
- Rectal temperature at pulling, lung auscultation score at pulling and lung lesions at slaughter may not be accurate for BRD case definition as single, independent measures.
- Blood metabolomics can detect animals with visual signs of BRD with 85% accuracy, or with visual signs of BRD confirmed by high rectal temperature and lung auscultation score with 81% accuracy.
- Blood metabolomics can detect animals with high rectal temperature, high lung auscultation scores, and lung lesions at slaughter with 69, 64, and 71% accuracy, respectively.

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