EFFECT OF BOVINE RESPIRATORY DISEASE INFECTION ON THE METABOLIC PROFILES OF BEEF STEERS

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THE EFFECT OF BOVINE RESPIRATORY DISEASE INFECTION ON THE METABOLIC PROFILES OF BEEF STEERS

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CHAPTER I

INTRODUCTION

Bovine respiratory disease (BRD) is the most common feedlot disease in North America. Over 14% of cattle in feedlots are affected by BRD (NAHMS, 2000). The combination of environmental changes and pathogens that newly weaned cattle are exposed to upon arrival to a feedlot greatly contributes to BRD outbreaks. Bovine respiratory disease detection and treatment is made more complicated by the multiple viral and bacterial pathogens that can act as disease factors for BRD infection. Bovine respiratory disease affects both the respiratory tract and the whole animal. The activation of the body's immune system and inflammation response affects other metabolic pathways and triggers decreased performance, thus reducing value.

Bovine respiratory disease affects a wide range of performance and carcass variables. Decreased average daily gain (ADG), as well as decreased hot carcass weight (HCW), marbling scores, and fat thickness have all been reported (Gardner et al., 1999; Montgomery et al., 2009). Because of the decreased carcass value, and the costs associated with treatment (e.g., drug cost, labor), cattle treated for BRD return between $46.64 to $291.93 less than healthy cattle, with returns decreasing as the number of treatments increases (Fulton et al., 2002).

Bovine respiratory disease diagnosis currently depends on the observation of clinical signs. Clinical signs include increases in respiratory rate, poor respiratory character, depression, nasal discharge, decreased rumen fill, depressed feed intake, and high rectal temperature (>39.7^{o}C) (Apley, 2006; Duff and Galyean, 2007). However, often by the time these symptoms are noticed, it may be too late for effective treatment. In fact, it has been shown that lung lesions, which are a common sign of BRD infection at slaughter, were present in 60.6% of animals never treated for BRD (Schneider et al., 2009). There are no fast, objective diagnostic tests currently available to detect BRD in high risk cattle. Cattle at high risk for BRD are usually from an unknown origin, recently weaned, and are from cow-calf operations that do not utilize consistent vaccination (NAHMS, 1997). By examining how BRD affects the whole body metabolism, and specifically how measurable plasma metabolites change, possible biomarkers can be identified that could aid in the diagnosis of BRD.

There have been many ways suggested to better diagnose BRD. The identification of one biomarker through the traditional methods has been suggested, although with varying results (Duff and Galyean, 2007). In addition, other nontraditional methods, like rumen temperature boluses, have been applied to the problem of prediction of BRD infection (Burciaga-Robles et al., 2009). However, a new analytical science has been developed in the past 10 years that may greatly change how we identify metabolic changes due to disease – metabolomics, also known as metabonomics. The term metabolomics was first coined in 1999 (Nicholson et al., 1999), and is defined as "the quantitative measurement of the dynamic multiparametric metabolic response of living systems to pathophysiological stimuli or genetic modification." In plainer terms,

metabolomics is the study of all the small molecules of a biological fluid or tissue (Nicholson et al., 1999). Metabolomics allows for the identification and quantification of small metabolites of various compound classes to occur at one time, in one assay, instead of multiple assays on the same sample. By utilizing analytical chemistry, like gas chromatography coupled with mass spectrometry, biomarkers of a wide range of molecules, from amino acids to sugars, can be identified. This could greatly speed up BRD biomarker identification. Metabolomics has not yet gained popularity outside of classical health research on humans and laboratory animals, but has great potential for usefulness in animal health research.

The present experiment was conducted to examine the effects of BRD infection on the plasma metabolome of beef steers. The examination of plasma metabolites may allow for the identification of possible BRD biomarkers that could make diagnosis quicker and more objective. The quantification of selected potential biomarkers could then be performed to establish concentrations that could denote the early stages of BRD infection.

CHAPTER II

REVIEW OF LITERATURE

Introduction

Bovine respiratory disease (BRD) causes major physiological changes to occur within the immune system and the metabolism of animals during infection. These changes are caused by the infection of both viral and bacterial pathogens, along with the stress of transportation and commingling of calves from multiple sources. The responses of the immune system and metabolism cause changes in blood metabolites, such as proteins, cytokines, and other small metabolites that have potential to be used as indicators of BRD infection. The detection of changes in metabolites have been mainly done using traditional laboratory methods; however, the advent of metabolomic analytical platforms, that allow for the detection of multiple metabolite compounds at one time, may provide better identification and quantification of BRD biomarkers.

Bovine Respiratory Disease

BRD is the major cause of morbidity and mortality in feedlots today. It is caused by the introduction of both viral and bacterial pathogens to a stressed calf upon entrance to feedlots or other settings where commingling and transportation of various calves

occurs. The effects of BRD are not just on the respiratory tract – rather, the disease affects the metabolism of the whole body by activating the immune response as well as affecting mineral status, energy metabolism, and protein metabolism.

Pathogenesis

Bovine respiratory disease results from the combination of pathogen exposure and stress. Bovine respiratory disease is most common in newly received feedlot cattle that are highly stressed due to transport and commingling, and are being exposed to a host of new pathogens, both viral and bacterial. BRD can be caused, or at least affected by both preweaning and postweaning factors. Early nutrition, temperament, and health management (i.e., vaccines) preweaning can help to deter the development of BRD further down the production line. Postweaning factors, such as transportation stress, commingling, and management techniques such as castration, receiving diets, and metaphylatic drug dosing can also affect the development of BRD (Duff and Galyean, 2007). Although management can decrease the risk of BRD development, the combination of unavoidable stress and pathogen exposure can be too much for the calf's host defenses to overcome.

Pathogens responsible for BRD infection include both viruses and bacteria. Common viral pathogens include bovine viral diarrhea virus (BVDV), bovine herpes virus-1 (BHV-1), bovine respiratory syncytial virus (BRSV), bovine coronavirus (BCV), and infectious bovine rhinotracheitis virus (IBRV). Although some of these pathogens, such as BVDV, may not initially appear to have direct effects on the respiratory system, they cause respiratory problems by affecting the whole body's immune status (Ellis, 2001). The decline in immune status then allows bacterial infections to gain hold in the

respiratory tract. BVDV has gained much of the attention mainly due to its prevalence. Vaccines have been developed for most strains of BVDV; however, even vaccinated calves face some risk of infection because of the multiple strains of BVDV. More than one virus is usually isolated from cattle diagnosed with BRD, once again indicating the complexity of the disease (Duff and Galyean, 2007).

Bacterial pathogens usually include a combination of *Mannheimia* (*Pasturella*) *haemolytica*, *Pasturella multocida*, and *Histophilus somni* (*Haemophilus somnus*) (Ellis, 2001; Apley, 2006). These gram negative bacteria all produce lipopolysaccharides (LPS) and/or lipooligosaccharides as pathogenic factors (Corbeil, 2007; Dabo et al., 2007; Rice et al., 2007). Of the major bacterial pathogens, *M. haemolytica* is generally considered to be the most prevalent and pathogenic. Interestingly, *M. haemolytica* is naturally found in the upper respiratory tract as a native bacterium, but acts as an opportunistic pathogen. It does not become pathogenic unless the immune system becomes compromised, such as during times of stress or infection. *M. haemolytica* produces not only a lipopolysaccharide virulence factor, but also a ruminant specific leukotoxin that targets ruminant leukocytes, which greatly enhances its virulence (Zecchinon et al, 2005).

Another less known bacterial pathogen is *Mycoplasma bovis.* This mycoplasma has recently become the subject of increased research especially in North America. In recent histopathogolocial evaluations of cattle diagnosed with BRD in Canadian feedlots, *M. bovis* was second only to *M. haemolytica* in prevalence, and actually was identified more often than *M. haemolytica* in cattle who were classified as chronically BRD infected (Booker et al., 2008).

Metabolic Response

Most research on metabolite changes in BRD has focused on the acute phase proteins, such as serum amyloid A (SAA), fibrinogen, and most commonly haptoglobin. Focus has also been put on characterizing the entire acute phase response, which includes not only the acute phase proteins, but inflammatory cytokine production as well (Baumann and Gauldie, 1994). Research has been limited on the effects of BRD infection on other, non-protein, non-immune response related metabolites, such as amino acids. Experiments that examine the effects of BRD include both controlled infections, where pathogens are known, as well as natural feedlot infection scenarios (Buckham-Sporer et al., 2008; Gershwin et al., 2005). In this review, if the causative pathogens are known, they will be stated along with metabolite changes.

In order to provide a good background on metabolic response to BRD infection, induction of the acute phase response (i.e. acute phase protein levels), activation of the immune system, and changes in small non-protein metabolite levels will be reviewed. The detection of a biomarker in serum that is indicative of BRD infection would greatly help BRD treatment and diagnosis. Accordingly, the main objectives of some of the trials discussed within this review involve the determination of possible BRD biomarkers.

Acute Phase Response

The acute phase response is a non-specific inflammatory reaction to an infection or injury. It is initiated at the site of infection, usually by tissue macrophages or blood monocytes. These immune cells then stimulate the release of cytokines, which initiate a signal cascade that causes the production of cortisol via the adrenal-pituitary axis, which, in concert with other cytokines, eventually results in the production of the acute phase proteins (serum amyloid A, fibrinogen, and haptoglobin) being produced and released by

the liver. The acute phase response has been shown to be triggered not only by infection, but also by stress. The acute phase response serves as an important regulator of the defense response, initiating fever, metabolic changes, production of immune cells, and host defense activation which will eventually result in the destruction of pathogens (Baumann and Gauldie, 1994).

Cattle infected with some form of BRD usually have increased concentrations of serum amyloid A (SAA). In calves that were challenged with BVDV, MH, or a combination of the two, all groups of infected calves showed increased SAA levels after infection (Ganheim et al., 2003). Calves challenged with the MH infection alone reached maximum SAA concentrations at 1-2 days post infection, while BVDV-challenged calves reached a maximum at 8-9 days after infection. Interestingly enough, calves who received both BVDV and MH infection exhibited a biphasic response, with 2-3 days between peaks. BVDV/MH calves also had the most days with higher than baseline levels of SAA (Ganheim et al., 2003).

Increases in SAA levels were also seen in other models of BRD (Heegaard et al., 2000; Carroll et al., 2009). In a trial where calves were infected with BRSV, SAA levels were elevated in all but one of the infected treatment group calves. The SAA levels of BRSV infected calves reached a peak of 5-7 times greater than control calves between day 5-8 post infection (Heegaard et al., 2000). Cattle injected with a dose of lipopolysaccharide (LPS; which can be used as a model of bacterial infection) also showed increases in SAA levels after infection as soon as 7 hours after initial dosing (Carroll et al., 2009). These results indicate that bacterial infections, or intravenous doses of LPS, initiate the acute phase response much quicker than viral infections. Bacterial

infections cause an increase in serum amyloid A levels within 36 hours after infection, while viruses can take up to 9 days after infection for SAA levels to peak.

Fibrinogen levels, like SAA levels, also increase after infection with BRD pathogens. Calves infected with a viral pathogen (BVDV), had increased fibrinogen levels compared to control calves, with maximum levels reached at 8-9 days after viral inoculation. Comparatively, calves that received just a *M. haemolytica* inoculation showed increased fibrinogen levels within 24 hours post infection. When cattle were first dosed with a virus, and then 5 days later given a *M. haemolytica* inoculation, fibrinogen levels did not increase in the five days between the inoculations, but reached a maximum 3-5 days after *M. haemolytica* inoculation. The viral-bacterial pathogen calves did have a greater overall number of days with elevated (as compared to normal control values) fibrinogen levels than those calves with only a viral infection (Ganheim et al., 2003). These results indicate that like SAA levels, fibrinogen levels increase more rapidly in cattle with bacterial infections than those with just viruses.

Berry et al. (2004) showed that feedlot cattle treated multiple times had greater fibrinogen levels than cattle never treated or treated only once. Different pathogens affect levels of fibrinogen – Nikunen et al. (2007) isolated pathogens from cattle naturally infected with BRD, and observed that only cattle from which *P. multocida* was isolated had increased fibrinogen.

Haptoglobin has received the most attention of the three major acute phase proteins. Haptoglobin, like SAA and fibrinogen, increases in serum concentrations after infection. Haptoglobin has been shown to increase following a bacterial challenge with *M. haemolytica*; however, a solely viral infection with BHV-1 did not trigger an increase

(Godson et al., 1996). Additionally, increases in haptoglobin were seen in cattle intravenously injected with lipopolysaccharides from *Escherichia coli*, another gram negative bacterium (Jacobsen et al., 2004). Like SAA and fibrinogen, in cattle infected with just *M. haemolytica*, haptoglobin levels peaked at around 2 days post infection, while haptoglobin levels in virally infected cattle (BVDV) did not reach peak haptoglobin levels until day 9 post infection (Ganheim et al., 2003).

In a trial where cattle were infected dually with BHV-1 and *M. haemolytica*, haptoglobin levels also increased post infection (Aich et al., 2009). The haptoglobin levels were elevated in both cattle that eventually survived infection and those that died (Aich et al., 2009). Haptoglobin levels were also elevated in cattle that were infected with BRSV (Grell et al., 2005). Multiple studies have reported that while elevated levels of haptoglobin were seen in cattle that were treated for BRD in a commercial setting (Berry et al., 2004; Burciaga-Robles et al., 2009), haptoglobin levels were not useful for predicting the number of treatments per animal that would be required (Burciaga-Robles et al., 2009).

The activation of the acute phase response, and the subsequent release of acute phase proteins into the serum is a result of infection with BRD pathogens. However, there is still variability in how much the concentrations of these proteins change, which confounds the use of them as BRD infection indicators. Further research is needed in order to validate the acute phase proteins as biomarkers.

Cytokines

Obviously, any infection or injury to the body will cause the activation of the acute phase response, which involves important molecules besides the acute phase

proteins. Cytokines have been shown to increase with infection, although the exact cytokines differ depending on the nature of the infection (viral, bacterial, etc.). Cytokines are small molecules released by immune cells in response to various stimuli, including inflammation and stress, and signal physiological changes. The release of some cytokines is considered part of the activation of the acute phase response (Baumann and Gauldie, 1994).

Accordingly, increases in cytokine concentrations have been shown to occur in BRD challenged cattle. Burciaga-Robles et al. (2010) reported that cattle infected with *M. haemolytica* had increased levels of tumor necrosis factor alpha (TNF-α), interleukin 1-beta (IL-1β), and interferon-γ (IFN-γ). Similarly, after intravenous dosing with LPS, blood concentrations of TNF-α, interleukin-6 (IL-6), IL-1β, and IFN-γ were elevated (Carroll et al, 2009).

Virally infected cattle have slightly altered cytokine expression as opposed to solely bacterial infections. BRSV infected cattle had increases in IL-6 and IFN-γ (Grell et al., 2005), while cattle infected with BVDV had a cytokine profile with heightened levels of TNF-α, IL-1β, as well as IL-6. Interestingly, cattle that were infected with both BVDV and MH saw increases only in TNF-α, IL-6, IFN-γ, but not IL-1β, even though IL-1β concentrations were high in separate viral and bacterial infections (Burciaga-Robles et al., 2010).

The function of each of these cytokines is well established. IL-1β, TNF-α, and IL-6 are activated in the first response line of an immune challenge, and their release increases inflammation and acts on the liver to induce the acute phase response. IFN-γ's

major function is the activation of macrophages, which can also be responsible for inflammation (Kindt et al., 2007).

Elevated cytokine levels are a good indication of infection, and may serve a role as indicators of disease. However, because of the differences of cytokine profiles between pathogens, as well as a lack of specificity for respiratory disease, other options for BRD biomarkers need to be explored.

Energy Metabolism

There have been few studies that have examined the effects of infection on small, non-mineral, non-protein plasma metabolites. Montgomery et al. (2009) examined how plasma metabolites at arrival changed in receiving heifers depending on the number of treatments for apparent BRD. These heifers had decreased glucose levels, which could be the result of a hypoglycemic effect due to disease challenge, and/or reduced feed intake due to depression (Montgomery et al., 2009). Conversely, glucose levels increased when cattle were infected with BHV-1 and *M. haemolytica* (Aich et al., 2009). These differences may be due to differences in pathogen load, as well as diet and relation of feeding time to sampling. Lactate levels also declined in cattle treated for BRD (Montgomery et al., 2009).

Lactate has been discussed as a possible BRD biomarker. Montgomery et al. (2009) saw a decline in lactate levels as number of BRD treatments increased. The decrease in lactate levels with the number of BRD treatments seen in the Montgomery trial is in opposition with another study, where BRD infected calves had increased levels of lactate as severity increased. In this trial, high lactate levels also correlated with increased mortality (Coghe et al., 2000). One explanation for the variability in lactate

levels is that the cattle in the Montgomery trial may not have had severe enough disease to see an increase in lactate. The oxygen transport chain has enough backup steps to continue to provide oxygen to tissues unless disease problems become severe (Coghe et al., 2000). Lactate has also been indicated as a biomarker for the prediction of viral-bacterial infection – lactate levels were higher in cattle that died of a combination BHV-1 and *M. haemolytica* infection than those that survived (Aich et al., 2009).

Protein Metabolism

Protein metabolism is affected by disease. For example, cattle that were injected with a dose of lipopolysaccharide (LPS), showed decreases in plasma levels of methionine, threonine, leucine, isoleucine, phenylalanine, tryptophan, glycine, serine, asparagine, and tyrosine, while alanine increased (Waggoner et al., 2009b). In a similar trial, decreases were seen in threonine, lysine, leucine, phenylalanine, tryptophan, asparagine, ornithine, and glutamate, although alanine again increased (Waggoner et al., 2009a). Increased valine was also interpreted as a possible biomarker of concurrent BHV-1 and *M. haemolytica* infection (Aich et al., 2009).

In general, the activation of the immune system in an infection causes a decrease in most plasma amino acid levels. This is due to the increased need for production of immune system cells, such as leukocytes, which can require high levels of specific amino acids (Colditz, 2002). Additionally, the activation of the acute phase response and the subsequent production of acute phase proteins also heightens the need for amino acids and may reduce available plasma amino acid levels (Sandberg et al., 2007). Amino acids are often transported from the muscle to the liver for this specific purpose. Degradation of these amino acids from muscle can also be induced by disease, and not only are these

amino acids used for production of proteins or immune cells, they can also be excreted, resulting in further nitrogen loss and muscle wasting (Powanda and Beisel, 2003). Also, as reduced intake is considered a clinical sign of disease (Duff and Galyean, 2007), less protein intake via feedstuffs exacerbates the effects of increased nitrogen usage by the body in order to mount an immune response. Protein metabolism is where reductions in average daily gain and body weights due to disease come into play – in order to meet the demands of the acute phase response and immune system, protein requirements greatly increase. Unless amino acids are supplemented into the diet, muscle protein will be catabolized to synthesize plasma proteins (Obled et al., 2003).

Conversely to the results above, amino acid levels were shown to be elevated after BHV-1 infection (Aich et al., 2007). It is possible that different disease factors, such as viral or bacterial, can affect nitrogen metabolism differently. Increased total plasma N concentrations have also been shown to be greater in cattle treated for BRD than in those that were not (Montgomery et al., 2009). Cattle challenged with IBRV also had an increase in total plasma proteins, increased serum N, while excretion of N increased, indicating that disease challenge increased N turnover and affected how N was utilized by tissue (Orr et al, 1988). Overall plasma nitrogen and protein increases are most likely due to the proliferation of the acute phase proteins and immune cells and molecules.

The variability of the effects of BRD on amino acid and protein metabolism may be due to the exact disease model used. LPS injections, which are a model of bacterial infections, tend to decrease amino acid concentrations, whereas viral infections have shown to cause an increase in serum amino acids. The variability of pathogens that can cause BRD also convolutes how BRD affects metabolism, and thus makes the search for

a biomarker of BRD more challenging. There are, however, emerging technologies that may hold the key to identifying BRD biomarkers.

Metabolomics

Metabolomics is a discipline that may help in the search for BRD-related biomarkers. Metabolomics is considered the final "omics" science – the last step in the cascade that begins with genomics, proceeds to proteomics, and concludes with metabolomics. Metabolomics focuses on small metabolites found in a specific biological fluid, like blood, urine, saliva, or tissues such as muscle or liver. Metabolomics is performed using analytical chemistry platforms like mass spectrometry and nuclear magnetic resonance (NMR). Examining changes in metabolite profiles, also known as metabolomes, of biological fluids of BRD cattle could expand knowledge of the physiological changes that occur during BRD infection, as well as help in the detection of biomarker(s) of disease.

History

The field of metabolomics is relatively new to the biological sciences with the term first being coined in the late 1990s (Oliver et al., 1998; Nicholson et al., 1999). The study of small metabolites has been around for many years – some have dated the earliest example of metabolomic research to Linus Pauling and his research in the 1960s (Vinayavekhin et al., 2009). However, what makes the new field of metabolomics special is not only the analytical methods used, but also the advent of bioinformatics software that can analyze large multivariate data sets.

The field of metabolomics can be divided into separate disciplines, all of which fall under the metabolomics umbrella. There has been some debate about the two major

terms; metabolomics and metabonomics. Some propose that these two terms are interchangeable (Madsen et al., 2010), while others have defined the two separately, with metabolomics defined as the identification and quantification of all the metabolites in a biological system (Fiehn et al., 2002), and metabonomics defined as the dynamic modeling of changes in a system due to biological stimuli (Nicholson et al., 1999; Dunn et al., 2005). Recently, the researchers who were at the forefront of the development of metabonomics/metabolomics published an article stating that the two could be used interchangeably, as their separate meanings had become archaic (Nicholson and Lindon, 2008). This paper will use metabolomics as the overall term to broadly describe the research of the field.

Metabolic profiling focuses on the quantification of a specific pathway or a specific group of compounds, all of which are known. Related to metabolic profiling is targeted profiling, which usually involves the quantification of a few known compounds in the same chemical class (Dettmer et al., 2007). Metabolic fingerprinting focuses on the full metabolic profile, also known as the whole metabolome of the tissue or biological fluid. From these fingerprints, samples can be classified into groups and comparisons among groups performed. In addition, this "global" analysis involves the identification of the metabolites that are causing the metabolome changes, which are usually unknown at the start of an experiment (Fiehn, 2002; Orešič, 2009). Metabolic footprinting is another term used to describe the evaluation of the metabolites present in spent growth media of cultured cells. This method is useful because it does not require the extraction of intracellular metabolites (Dunn et al., 2005).

Applications of metabolomics are spread across the biological sciences. One of the first uses of metabolomics was to examine compound toxicity and how those toxins created metabolic perturbations in the NMR profiles of mode l organisms (Holmes et al., 1998). Metabolomics has also been used as a way to measure how genes can affect multiple metabolic pathways – metabolomics allows for the examination of many biological circuits at one time (Fiehn, 2002). The diagnosis and prediction of diseases has also benefited from metabolomics usage (Feng et al., 2009).

Methods

Metabolomic analyses are performed using any number of analytical chemistry methods. The two most common methods are ^{1}H nuclear magnetic reasonance spectroscopy (NMR) and mass spectrometry (MS) (Vinayavekhin et al., 2010). NMR is usually utilized alone, while MS is commonly coupled to a preceding separation technique, such as gas chromatography (GC) or liquid chromatography (LC) (Dettmer et al., 2007). The metabolomics workflow is similar among the treatments, although there are differences in sample preparation depending upon the analysis technique.

Sample preparation usually involves multiple steps, both through extraction or derivitization depending on the analysis type. One of the most important steps, however, is the halting of all enzymatic activity when the sample is taken. This can be done through freezing or the addition of enzymatic stopping reagents (Viant, 2009). The level of sample preparation beyond halting of enzymatic activity depends greatly on the analytical method used. The extraction of the small metabolite fraction is also important to remove interfering components, such as proteins. This process is done through the

addition of organic solvents, such as acetonitrile or methanol to the sample, which causes precipitation of the protein portion (Want et al., 2005).

After extraction of the samples, various platforms of analytical chemistry, such as mass spectrometry coupled with a chromatographic precursor, or NMR analyze the sample. These platforms are explained in further detail below. These platforms are important; however, many times the data analysis portion of metabolomics research is the limiting factor.

Mass Spectrometry

Mass spectrometry greatly predates metabolomics; mass spectrometry was first discovered in 1897 when Sir Joseph Thomson measured the mass to charge (*m/z*) ratios during his cathode ray experiments (Dass, 2001; Downard, 2004). However, modern mass spectrometry did not catch on until the 1940s, and developments since then, such as new ionization techniques (electrospray ionization (ESI)), as well as coupling with separation techniques such as gas chromatography and liquid chromatography, have helped mass spectrometry become an analytical workhorse of the biological sciences.

Mass spectrometry is popular because of its high sensitivity and versatility. It can analyze and produce mass spectra for most, if not all compound classes including all elements, and has great versatility in what type of samples it can analyze (gas, liquid, polar, etc.). Additionally, mass spectrometers are very sensitive and can detect very low concentrations of compounds in most samples (Dass, 2001; Dettmer et al., 2007).

All mass spectrometers, no matter the type, have three basic components; and ion source, mass analyzer, and ion detector. The ion source introduces molecules into the rest of the mass spectrometer, while also adding a charge or converting the molecules to

ions. The ions then enter the mass analyzer, where they are separated according to mass and charge. Finally, the charged ions pass to the ion detector, where the mass spectra are recorded. The entirety of the mass spectrometer is operated under a vacuum to prevent the collision of the charged ions with other gas molecules (Downard, 2004).

There are many different types of mass spectrometers, and one of the major differences between the types is how the molecules are ionized. Types of ionization include matrix assisted laser/desorption ionization (MALDI), atmospheric pressure chemical ionization (APCI), electrospray ionization (ESI), electron ionization (EI), among others too numerous to list here (Griffiths and Wang, 2009). EI was generally considered the traditional method of ionization techniques (Downard, 2004), but other types such as MALDI and ESI are becoming more commonly used (Griffiths and Wang, 2009). Mass spectrometers can also be classified by their mass analyzer type. Common mass analyzers include time-of-flight (TOF), single or triple quadrupole, and fourier transform (FT) analyzers (Dass, 2001). Triple quadrapole analyzers are generally considered the standard workhorse for small metabolite analysis (Lu et al., 2008), although TOF and FT are also used.

Separation Methods

The use of gas chromatography coupled to mass spectrometry (GC-MS) has greatly increased since the year 2000. This is due to the low cost of GC-MS compared to other methods such as NMR or LC-MS, along with high resolution and repeatability, high sensitivity and the ability to detect a wide range of molecules. However, GC-MS also takes longer compared to faster high thorough-put methods like NMR, and requires a derivitization step (Kopka, 2006; Shulaev, 2006).

One of downsides of GC-MS platforms is that most samples require a derivitization step, which is necessary to increase thermal stability and volatility of polar metabolites (Dettmer et al., 2007). This is usually done through trimethylsilylation of the metabolites. Roessner et al. (2000) tested multiple trimethylsilylation reagents, and found that *N*-methyl-*N*-(trimethyl)trifluoroacetamide (MSTFA) gave the most accurate results over a wide range of chemical compound classes. This same trial also examined methoximation procedures – methoximation works to stabilize sugars and prevent them from forming ring structures. These derivitization procedures have become common among GC-MS based metabolomic studies across a wide range of disciplines, from studies involving plant metabolites (Choi et al., 2010) to levels of serum metabolites in humans (Yan et al., 2009).

Liquid chromatography attached to mass spectrometry (LC-MS) is another important metabolomic platform. LC-MS, while similar in name to GC-MS, does not usually require derivitization – however, its libraries are limited in comparison to NMR and GC-MS (Shulaev, 2006). Most LC performed today is high performance liquid chromatography (HPLC), where the mobile phase liquid is injected under high pressure onto the solid-phase column (Ardrey, 2003). Chromatography, especially HPLC, is becoming more popular in metabolomics as this technique is able to analyze any compound that can be dissolved in liquid (Allwood and Goodacre, 2010).

Nuclear Magnetic Resonance

NMR was the original analytical method used when the term ‘metabonomics’ was coined (Nicholson et al., 1999). NMR was developed in the mid-1940s, and has been used for many years as a potent molecular structure elucidation technique (Günther,

1995). NMR detects structures by measuring the interaction of radiofrequency electromagnetic radiation with the nuclei of molecules while in a strong magnetic field (Rahman and Choudhary, 1996). Each part of a molecule has a separate absorption band on the spectra that give a specific signal depending on its structure. For example, a methanol molecule will have separate signals for the OH and CH_3 groups, and the functional groups will have a signature display, such as single peaks, doublets, and so on (Canet, 1996). This specificity of signals allows for the structural identification of various molecules and compounds.

NMR is still highly useful in the realm of metabolomics research today. It is one of the quickest analytic methods, requiring very little sample preparation, and is non-destructive to samples. Basically, for biofluids such as serum or plasma, no processing is required except for the addition of a less than 1% saline solution for dilution purposes (Beckonert et al., 2007). However, the cost of NMR can be prohibitive, especially to many smaller laboratories. Additionally, NMR spectra can be very convoluted, due to the lack of separation techniques prior to analysis, and also is unable to detect metabolites present at low concentrations (Shulaev, 2006).

Advances have been made in NMR technology that can fix some of these convolution problems. Notably, the development of two-dimensional NMR has increased specificity. Two-dimensional NMR records data from two time domains – the first collection, as discussed above, followed by successively incremented delay. This enables the detection of previously undetectable properties, such as spin-spin coupling and resonance frequencies (Günther, 1995). Two-dimensional NMR separates the

overlapping resonances that cause convolution in regular, one-dimensional NMR (Ludwig and Viant, 2009).

NMR has been used in all aspects of metabolomics. From metabolic profiling of humans with bipolar disorder (Sussulini et al., 2009), to differences in the serum metabolome of patients with leukemia (Macintyre et al., 2010), NMR has been beneficial. NMR has been the most utilized form of metabolomics analysis in nutritional metabolomics (Scalbert et al., 2009). Metabolic footprinting, or extracellular metabolomics, also utilizes NMR technology (Behrends et al., 2010).

Disease Detection and Biomarkers

Although metabolomics first began in the plant and bacterial realms, it has quickly spread to mammalian systems as a way to discover biomarkers of various diseases. Metabolomics has been utilized in a wide range of diseases in both humans and laboratory animal models. Diseases have ranged from neurological disorders such as motor neuron disease in humans (Rozen et al., 2005) to parasite infestations in mice (Li et al., 2008). Both MS and NMR analytical methods have been used in mammalian systems, so investigations using both of those research methods will be discussed. The studies discussed also use a variety of biological fluids and tissues, although urine and blood are most commonly used.

The identification of early biomarkers of cancer has been an area of fairly rapid development. Because of its high death rate, pancreatic cancer has been the subject of much biomarker research, with biomarkers being identified in plasma (Urayama et al., 2010) and saliva (Sugimoto et al., 2010). Biomarkers of colorectal cancer have also been identified in serum (Ritchie et al., 2010; Ludwig et al., 2009), as well as in tissues from

the digestive tract (Chan et al., 2009; Denkert et al., 2009) and urine (Feng et al., 2009). Breast cancer biomarkers have also been under scrutiny, with tissue (Sitter et al., 2009), saliva (Sugimoto et al., 2010) and urine (Chen et al., 2009) all being examined. Biomarkers of lung cancer, which is the leading cause of cancer related deaths, have also been found in urine (Matsumura et al., 2010) and tissue (Fan et al., 2009).

Biomarkers determined by metabolomic techniques have also been found in subjects with lung injury and damage. In mice dosed intratracheally with inflammatory cytokines in order to simulate lung injury, a decline in energy metabolites in lung tissue was observed when examined via NMR (Serkova et al., 2008). The severity of the lung inflammation/damage was also able to be distinguished using the NMR spectral profiles. (Serkova et al., 2008). NMR was also used to examine bronchial-alveolar lavage fluid from children with chronic cystic fibrosis who had either high or low levels of inflammation. Metabolic profiles easily distinguished between the two inflammation groups, with high inflammation patients having increased complexity and higher concentrations of most metabolites, while low inflammation patients had overall lower concentrations of all metabolites (Wolak et al., 2009).

Metabolomics has also been used to examine different bacterial infections and their effects on metabolism and metabolite profiles. Feng et al. (2008) injected mice with a combination of lipopolysaccharide and galactosamine into the peritoneal cavity. The plasma profiles of small metabolites, including energy substrates and amino acids, were different between the control and injected mice. Similarly, mice infected with bacterial meningitis could be distinguished from control mice by examining NMR profiles of cerebrospinal fluid (Himmelreich et al., 2009). Urinary metabolite profiles can also

distinguish between lung infections and controls using two separate bacteria, *Staphylococcus aureus* and *Streptococcus pneumonaie*. The differences in the urinary metabolic profiles were so definite that not only could non-infected versus infected mice be separated, but the two different bacterially infected mice groups could also be distinguished (Slupsky et al., 2009).

Viral infections can also change the metabolome of biological fluids and tissues. Mice injected with lymphocytic choriomeningitis virus (LCMV) had differing metabolic profiles from control mice, including a decline in the activity and levels of metabolites from the Kreb's cycle in the blood (Wikoff et al., 2009). Xue et al. (2009) also observed that serum profiles could distinguish between and be used to classify patients suffering from Hepatitis B virus infections.

Current Applications in Animal Science

Metabolomics in production animals has lagged behind metabolomics research in the plant, bacterial, lab animal and human areas. Few papers have been published regarding changes in farm animal metabolites due to disease or nutrition. This research has included examining the effects of feeding L-arginine on the serum metabolome in pigs (He et al., 2009), using metabolome profiling to detect growth hormone usage in racehorses (Kieken et al., 2009), and work on determining biomarkers of BRD mortality in beef cattle and effect of stress coupled with a viral infection (Aich et al., 2007; Aich et al., 2009). The use of anabolic steroids in cattle has also been shown to be able to be distinguished from control cattle via NMR metabolomics, indicating that using metabolic signatures for identification of users of anabolic steroids (whether human or performance animal) is feasible (Dumas et al., 2005). Milk metabolites have also been examined in

order to aid in selection of cows that can handle the stressors of early lactation more satisfactorily (Klein et al., 2010).

There are many applications for metabolomics in the animal sciences. Metabolomics could aid in the detection of drug toxicity, and also eventually decrease the number of laboratory animals euthanized for histopathological toxin screening, if toxin levels can be detected via non-invasive biological fluids (Jones and Cheung, 2007). Examining metabolic signatures could aid in genetic selection of animals better suited for varying climates or production schemes, as well as aid in identification of high disease risk animals in order to decrease unnecessary antibiotic usage. The identification of high risk cattle, or even the identification of early biomarkers for prevalent animal diseases, such as BRD, could greatly help the detection and treatment of BRD infected animals by allowing for earlier, objective, diagnosis.

Summary

BRD is the leading cause of mortality in North American feedlots, and affects a multitude of biological systems within the animals, including immune activation and energy/protein metabolism. The biological changes that occur with disease status, while not beneficial to the animal, may prove to be useful for identifying infected animals before the presentation of clinical signs. As has been shown in human diseases and model organisms, disease status can be differentiated and biomarkers identified using metabolomic techniques. When applied to BRD, metabolomics could allow for the discovery of early biomarkers of infection in high risk cattle, and lead to improved diagnosis and management.

CHAPTER III

USE OF METABOLOMIC TECHNIQUES TO DETERMINE METABOLIC CHANGES IN STEERS INFECTED WITH PATHOGENS OF BOVINE RESPIRATORY DISEASE

Abstract

Bovine respiratory disease (BRD) is the most costly disease in North American feedlots; however, diagnosis is subjective. Metabolomics, or the study of the total metabolic profile of a biological tissue or fluid, may provide a way for objective diagnosis. The objective was to identify biomarkers of BRD using metabolomic techniques (i.e., GC/MS). Twenty-four Angus crossbred steers were divided into four treatment groups in a randomized complete block design (n = 3 steers/block/treatment). Treatments were: 1) exposure to 2 BVDV persistently infected (PI) steers for 72 h (BVDV); 2) exposure to the 2 PI-BVDV steers for 72 h and intratracheal challenge with *Mannheimia haemolytica* on d 0 (BVDVMH); 3) intratracheal challenge with *M. haemolytica* on d 0 (MH); and 4) no challenge (CTRL). Blood samples were collected at -72, 12, 24, and 48 h of *M. haemolytica* challenge. Using a GC/MS platform, total metabolic fingerprints of plasma were obtained. Normalized abundance values were analyzed and means separated using Tukey's procedure (GeneSpring MS 1.2; Agilent

Technologies, Santa Clara, CA), and metabolites were identified using the NIST ’05 MS Database (NIST, Gaithersburg, MD). At 12 h after infection, the amino acids isoleucine decreased ($P = 0.003$) in BVDVMH and MH cattle, glutamic acid decreased in BVDVMH, BVDV, and MH steers ($P = 0.003$), and tyrosine decreased in MH steers ($P = 0.005$) compared with CTRL steers. Decreased plasma levels of threonic acid ($P = 0.040$) and citric acid ($P = 0.003$) were found in BVDVMH, BVDV, and MH cattle compared with CTRL. Mannitol levels were decreased in BVDVMH and MH steers ($P = 0.014$), and galacturonic acid ($P = 0.049$) and galactofuranoside ($P = 0.024$) were decreased in BVDVMH, BVDV, and MH cattle. Propanoic acid decreased in BVDVMH and BVDV steers ($P = 0.049$), while galactose decreased in BVDV and MH steers ($P = 0.037$). At 24 h post infection, 2-ethyl-3-hydroxypropionic acid ($P = 0.003$) was observed to decrease in BVDVMH and BVDV steers, while tyrosine ($P = 0.039$) and glycine ($P = 0.035$) decreased in only MH steers. Forty-eight hours after infection, increases in alanine ($P = 0.014$) in BVDVMH and BVDV steers and decreases of glycine ($P = 0.020$) in BVDVMH, BVDV, and MH cattle were observed. Phenylalanine ($P = 0.006$) increased in BVDV steers, but was decreased in both BVDVMH and MH steers. Valine ($P < 0.01$) and leucine ($P < 0.01$) were both increased in BVDVMH but decreased in BVDV steers. Other metabolites that changed included an increase in arabinose in BVDV steers, and a decrease in acetic acid in BVDV and MH steers. Changes in plasma amino acid concentrations were further examined through targeted analysis via GC/MS. In general, amino acids were observed to be decreased ($P < 0.05$) in BVDVMH and MH steers as compared to CTRL and BVDV steers. At 12 h after infection, glycine and isoleucine levels were decreased in BVDVMH steers compared with CTRL steers.

Threonine, serine, proline, methionine, glutamic acid, ornithine, lysine, and tryptophan concentrations were decreased ($P < 0.05$) in BVDVMH and MH steers compared with BVDV steers. Leucine concentrations were increased ($P = 0.001$) in BVDV steers compared with CTRL, BVDVMH, and MH steers. At 48 h after infection, methionine and 4-hydroxyproline levels were decreased ($P < 0.03$) in BVDVMH and MH steers compared with CTRL. Metabolomics has potential to identify biomarkers; however, further analysis and research is needed in order to confirm the identities of possible biomarkers.

Introduction

Bovine respiratory disease (BRD) is the most common disease in North American feedlots (NAHMS, 2000). It is caused by the combination of exposure to new pathogens and stressors that cattle encounter upon arrival at feedlots. Current diagnostic methods are highly subjective and are focused on using visual appraisal and body temperature (Duff and Galyean, 2007). There is a need for the development of definitive, objective diagnostic tests that can detect when cattle are in the early stages of BRD. One way that may help to identify novel diagnostic markers is metabolomics. Metabolomics examines all the metabolites of a biological fluid or tissue, such as plasma. By examining metabolites of BRD infected cattle, biomarkers of the disease may be able to be identified. Identification of biomarkers could greatly improve the diagnosis and treatment of BRD. We hypothesized that biomarkers of BRD challenged cattle could be identified using metabolomics. The objective of the experiment was to identify possible biomarkers of BRD infection using metabolomic techniques, and then to validate those changes using targeted analysis.

Materials and Methods

Animals

Twenty-four crossbred Angus steers (initial BW = 313 ± 31 kg) were housed at the Nutrition Physiology Research Barn, located at Oklahoma State University, Stillwater. Prior to the start of the experiment, all animals were considered healthy and tested seronegative to all pathogens used in this trial using paired serum samples prior to the start of the experiment. All procedures were approved by the Oklahoma State University Institutional Animal Care and Use Committee (Protocol # AG0616).

Treatments

Steers were randomly assigned to one of four treatments: 1) exposure to 2 bovine viral diarrhea virus persistently infected (PI) steers for 72 h (BVDV); 2) exposure to the 2 PI-BVDV steers for 72 h and intratracheal challenge with *Mannheimia haemolytica* on d 0 (BVDVMH); 3) intratracheal challenge with *M. haemolytica* on d 0 (MH); and 4) no challenge (CTRL). Steers were also blocked by weight, with equal numbers of each block represented in each treatment (3 steers/block/treatment). Steers challenged with BVDV were transported to the Willard Sparks Beef Research Center (3.2 km) where they were exposed to the 2 PI steers (BVDV Type 1b) for 72 h. *M. haemolytica* challenge was performed by intratracheally dosing steers with 10 ml of solution containing 6 X 10^9 CFU of *M. haemolytica* serotype A-1 (Buricaga-Robles et al., 2010). Control steers were dosed with 10 ml of phosphate buffered saline solution in the same manner. All *M. haemolytica* dosing occurred at the same time and same day. Blood samples were collected at -72, 12, 24, and 48 h of *M. haemolytica* challenge. Blood samples were

collected via jugular venipuncture (Lithium Heparin; Becton Dickinson Vacutainer Systems, Franklin Lakes, NJ) with an 18 x 1 gauge collection needle. Plasma was harvested and frozen until time of analysis.

Metabolomic Analysis

Extraction of protein from the plasma samples was done by use of Protein Precipitation Plates (Thermo Scientific, Rockford, IL). All samples were repeated in triplicate. One hundred and eighty microliters (µl) of acetonitrile was added to 60 µl of plasma sample in a 2 ml amber glass vial. One microliter of ribitol (1 mg/ml concentration) was added to serve as an internal standard for calibration purposes. The vial was then swirled and the solution was pipetted from the vial into an individual well on the precipitation plate. The plates were centrifuged in an Avanti J-E Centrifuge (Beckman-Coulter, Brea, CA) for three minutes at 600 x g. The clear supernatant was removed from the collection plate and pipetted into a new amber glass vial and dried completely under a stream of nitrogen.

After drying, a derivitization procedure modified from Fiehn and Kind (2006) was performed. Methoxyamine (MOX; Thermo Scientific, Rockford IL) was removed from cold storage and allowed to warm to room temperature. Twenty-five microliters of MOX was added to each dried sample. Vials were swirled and then loosely capped and incubated at 45^{o}C for 90 minutes. During the incubation time, N-Methyl-N-trifluoroacetamide (MSTFA; Thermo Scientific, Rockford, IL) was removed from cold storage and warmed to room temperature. After removal from incubation, samples were cooled to room temperature and 160 µl of MSFTA was added to each sample. Samples were then incubated for 30 minutes at 37^{o}C in a loosely capped vial.

After the sample cooled to room temperature, the sample was pipetted into glass GC inserts (Agilent Technologies, Santa Clara, CA) that contained a mixed hydrocarbon standard. The standard consisted of dodecane, heptadecane, eicosane, docosane, and pentacosane at individual concentrations of 400 nM in order to aid in retention time locking. Samples were injected onto a 30 m x 250 µm x 0.25 µm HP5-MS column (J & W, Agilent Technologies, Santa Clara, CA) in an Agilent 7890A GC, coupled with an Agilent 5975C inert XL EI Mass Spectrometer with a triple axis detector (Agilent Technologies, Santa Clara, CA). The sample was injected into the GC in splitless mode at a temperature of 250^{o}C, and the GC oven was programmed to rise from 50^{o}C to 315^{o}C at a rate of 10^{o}C/min. The flow of the helium gas carrier was set at 1 ml/min, with a total run time of 36.5 minutes. A solvent delay time of 9.8 minutes was selected, with the MS source temperature set at 230^{o}C, and the MS quadrapole mass analyzer temperature at 150^{o}C. The sampling rate was set at 3.5 scans/second, with a mass scanning range of 35-550 m/z.

Data Processing

Raw chromatographic and mass spectral data was deposited into Chemstation (Agilent Technologies, Santa Clara, CA), and was initially processed and deconvoluted using Automated Mass Deconvolution and Identification Software (AMDIS; National Institute of Standards and Technology [NIST], Gaithersburg, MD). The hydrocarbon standard mixture was used to create a retention time index library in AMDIS. This library was used to help calibrate the chromatograms and aid in the identification of compounds. The deconvoluted spectra were then imported into GeneSpring MS 1.2 software (Agilent Technologies, Santa Clara, CA) for statistical analysis.

Amino Acid Analysis

Plasma amino acid analysis was performed using the EZ:faast Free (Physiological) Amino Acid Analysis by GC-MS kit (Phenomenex, Torrance, CA). All reagents and materials for analysis were provided in the kit. One hundred microliters of plasma sample was combined with 100 µl of an internal standard solution containing N-propanol and 200 nM norvaline as the internal standard. This mixture was drawn slowly through a sorbent tip, after which 200 µl of wash solution containing N-propanol was also drawn through the sorbent tip. Two hundred microliters of eluting medium was drawn up into the sorbent tip – and the eluting medium was expelled along with the sorbent particles until all particles were removed from the tip. Following this step, derivitizing agents included in the kit were added to the mixture. After vortexing and following a one minute settling period, the upper organic layer was transferred via pipette to an autosampler vial and dried under nitrogen until almost dry (<10 min). The sample was reconstituted using the kit-provided reagent. The solution was transferred to an insert and capped for GC/MS analysis. The final sample was injected onto a 10 m x 0.25 mm Zebron Amino Acid column (Phenomenex, Torrance, CA) in an Agilent 7890A GC, coupled with an Agilent 5975C inert XL EI Mass Spectrometer with a triple axis detector (Agilent Technologies, Santa Clara, CA). Samples were injected via split mode with a 15:1 split ratio at a temperature of 250^{o}C, with the flow set at 1.1 ml helium/min. The GC oven was programmed to rise at 30^{o}C/min from 110^{o}C to 320^{o}C. The solvent delay time was 1.0 minute, and the MS source temperature was set at 230^{o}C and the MS quadrapole mass analyzer at 150^{o}C, with a sampling rate of 3.5 scans/second and a scan range of 50-500 m/z. Amino acid chromatograms and mass spectral data were integrated

using Chemstation. Compounds were quantitated using a standard curve derived from standards included in the EZ:faast kit.

Statistical Analysis and Compound Identification

In GeneSpring MS 1.2, the spectra were grouped by time point, and normalized using the standard MS setting. Each time point was evaluated individually. Once the data had been normalized, masses were then filtered by relative frequency. One-way ANOVA was performed on the filtered masses, and differences between treated and control cattle were examined. The samples were randomly organized. The significance level was set at 0.05. In time points where differences were observed between treated and control cattle, the Tukey post-hoc test was performed. The retention times of each significantly different metabolite between control and treatment groups were recorded for each sample. The spectrum of the peak at that retention time was examined and its identity confirmed in AMDIS using the NIST '05 Mass Spectral Database (NIST, Gaithersburg, MD) and the Golm Metabolome Database (Max Planck Institute of Molecular Plant Physiology, Potsdam, Germany).

Amino acid data was analyzed using the MIXED procedure of SAS (SAS Institute, Cary, NC) with repeated measures. Animal was the experimental unit and the independent variables in the model included treatment and time. Covariates included block, block by treatment, and animal within block by treatment. Random effects were block, treatment by block, and animal within treatment by block. The significance level was set at α=0.05. PDiff was used to calculated differences between LSMeans.

Results and Discussion

This challenge model has been shown to induce BRD infection (Burciaga-Robles et al., 2010). Increased rectal temperatures and increased levels of the cytokines IL-1β, IL-6, and TNF-α, which are all indicative of infection, were observed in cattle infected with BVDV, MH or their combination (Burciaga-Robles et al., 2010).

Principal Component Analysis. Principal component analysis (PCA) plots for all time points are displayed in Figures 1 through 4. PCA plots allow for the visualization of differences over the entire metabolic profile.

Prior to the start of the challenge (72 h prior to infection), there was little to no separation among the treatment groups (Fig. 1). This indicates that prior to the start of the experiment, all cattle had similar metabolic profiles. In addition, further statistical analysis of the metabolomes showed that no metabolites were significantly different among cattle prior to infection. This allows for the assumption that there were no biases among the treatment groups prior to infection and the start of the experiment.

Separation of the treatment groups became evident at 12 h after infection (Fig. 2). The CTRL and BVDV groups were easily distinguishable from the MH and BVDVMH groups, indicating differences existed between their metabolic profiles. The individual cattle in each group also clustered together in the same region, suggesting that changes in metabolite concentrations occurred between treatment groups and not within groups. There was considerable overlap between MH and BVDVMH steers, which may indicate that the profiles of these two groups changed in similar ways. The overlap of the treatments that included the bacterial infection suggests that *M. haemolytica* may have had a greater effect on the metabolic profiles than viral infection. This may also be due

to the faster response of the body's defense systems to *M. haemolytica* infection. *M. haemolytica* has been shown to activate the acute phase response and begin raising levels of the acute phase proteins within 24 h after challenge, while BVDV infection did not cause a response until almost 4 to 8 days after infection (Ganheim et al., 2003).

Interestingly, at 24 h after infection (Fig. 3), treatment groups were less distinguishable from each other than at either the 12 or 48 h time points. At 24 h, all treatment groups have considerable overlap with each other. This suggests that there were fewer changes in metabolite levels among groups at 24 hours after infection. This result was unexpected, as it was predicted that treatment groups would differentiate from each other at all time points after the challenge. There were still a few metabolites that significantly changed at this time point; however, these changes were not enough to trigger separation of the treatment groups in the PCA plot.

At 48 h post infection (Fig. 4), the trends of the PCA were similar to those of the 12 h after infection. The BVDVMH and MH treatment groups overlapped, while both the CTRL and BVDV groups were separated out from each other and the other treatment groups. This pattern suggests that *M. haemolytica* infection caused the metabolic profiles of BVDVMH and MH steers to change in a more similar manner.

The differences in the metabolic profile of the different treatment groups may also be due to the lag time between viral infection and activation of the acute phase response. Viral pathogens associated with BRD generally take 7 to 9 days to activate the body's defense responses, such as the acute phase response (Heegaard et al., 2000; Ganheim et al., 2003). It would follow then that the metabolic profile would also not be fully affected until that time. Changes were seen in some metabolites of BVDV versus CTRL

steers at all time points, so it is evident that viral infection at least began to affect metabolism.

Metabolites Identified by Metabolomic Analysis. The most common metabolites that were identified as significantly changing were amino acids and energy substrates.

12 h after infection. At 12 h post infection, the amino acids identified as changing in challenged cattle compared with CTRL were isoleucine, glutamic acid, and tyrosine (Table 1). Glutamic acid was lower ($P = 0.003$) across all treatment groups compared with CTRL cattle. Glutamate (glutamic acid) has been shown to decrease in cattle dosed with LPS, one of the major pathogens produced by *M. haemolytica* (Waggoner et al., 2009a). Tyrosine decreased ($P = 0.005$) only in MH cattle when compared to CTRL, which has also been noted to occur in cattle challenged with LPS (Waggoner et al., 2009b). Isoleucine was decreased ($P = 0.003$) in both BVDVMH and MH cattle compared with CTRL. As a branched chain amino acid (BCAA), isoleucine is important in protein metabolism. In most stressed states, such as infection, the BCAA are mobilized from muscle stores in order to be utilized for energy. However, the BCAA are quickly catabolized, meaning that their levels in blood are usually decreased compared to healthy animals (Harris et al., 2003; Tom and Nair, 2006), which was seen in this experiment for cattle challenged with *M. haemolytica*. The breakdown of BCAA is catalyzed by the rate limiting enzyme branched chained α-keto acid dehydrogenase (BCKAD), the activation of which has been shown to be increased by administration of inflammatory cytokines such as TNF-α in rats (Nawabi et al., 1990). TNF-α levels have been shown to be increased in BVDV and MH challenged cattle (Burciaga-Robles et al., 2010).

Threonic acid was also affected by BRD infection, with plasma levels increasing ($P = 0.040$) in BVDVMH, BVDV, and MH cattle versus CTRL (Table 1). Threonic acid, also known as threonate, is formed from L-dehydroascorbate (DHA), which is the primary oxidation product of the interaction of ascorbic acid and radical oxygen species (ROS; Simpson and Ortwerth, 2000). Elevated levels of threonic acid could be due to increased levels of radical oxygen species being scavenged by ascorbic acid, leading to increased oxidation products. Dwenger et al. (1994) saw improved respiratory function in sheep suffering from endotoxin induced lung inflammation when infused with ascorbic acid, indicating that ROS species are a mechanism in endotoxin infection, and increased levels of threonic acid may be an indicator of neutralization of these species.

Citric acid and other energy substrates such as galactose also decreased ($P < 0.05$) at 12 h after infection (Table 1). Citric acid decreased ($P = 0.003$) in virally challenged calves, as did propanoic acid ($P = 0.049$). Galacturonic acid and galactofuranoside decreased ($P < 0.05$) in all challenged cattle. Galactose decreased ($P = 0.037$) only in BVDV and MH steers. The production of immune cells can increase energy expenditure, which could lead to decrease levels of energy substrates in the blood. During times of inflammatory infection nutrients are often shunted from growth to the production of immune cells (Spurlock, 1997) increasing overall energy requirements.

24 h after infection. There were only three metabolites detected as changing at 24 h after infection (Table 2). In steers that were challenged with a viral pathogen, decreases ($P = 0.003$) in the plasma concentrations of 2-ethyl-3-hydroxypropionic acid were seen. This metabolite has been indicated as a possible marker of deficiencies in the catabolism of isoleucine in humans (Human Metabolome Database [HMDB]; Wishart et

al., 2009). As this biomarker was not indicated in steers challenged with MH, decreases in 2-ethyl-3-hydroxypropionic acid may be unique to viral infection.

In MH steers, the amino acids glycine and tyrosine were both decreased ($P < 0.04$) compared with CTRL (Table 2). The changes in only cattle challenged with MH again allude to the faster pathogenesis of bacterial infection. The dosing of cattle with LPS has shown to decrease levels of both glycine and tyrosine in plasma for at least 12 h after challenge (Waggoner et al., 2009b).

48 h after infection. Table 3 exhibits the metabolites that were different between challenged and control cattle. Alanine, glycine, phenylalanine, valine, and leucine were all detected as differing ($P < 0.05$) from CTRL. Alanine increased ($P = 0.014$) in BVDVMH and BVDV cattle, but had no change in MH steers. Interestingly, glycine reacted in the opposite manner, decreasing ($P = 0.020$) in both BVDVMH and BVDV cattle, but increased in MH steers. Phenylalanine, valine, and leucine all decreased ($P < 0.01$) in BVDVMH steers, but increased in BVDV steers. Phenylalanine also decreased ($P = 0.006$) in MH cattle. These changes indicate that amino acid metabolism is affected differently depending on the severity and type of infection, whether bacterial or viral. Decreased amino acid concentrations could also be due to increased use of amino acids for energy (Beisel, 1975). Amino acid concentrations have been shown to increase during a viral challenge with BHV-1 (Aich et al., 2007), and increased valine, in particular, was identified as a biomarker of BHV-1 and *M. haemolytica* infection (Aich et al., 2009). However, it has also been stated that the mobilization of amino acids for production of immune cells can decrease plasma concentrations (Colditz, 2002). Levels of amino acids were generally decreased at 48 h after infection for steers that had a

bacterial component of the challenge. This could be due to the faster action of endotoxin infection, which would cause an acute phase response, and thus deplete plasma levels of amino acids.

Acetic acid and arabinose were also affected ($P \leq 0.04$) by infection (Table 3). Acetic acid levels were lower ($P = 0.04$) in both BVDV and MH cattle, while arabinose was increased ($P = 0.024$) in BVDV. As seen at the 12 h time point, energy requirements change during inflammation and infection. Acetic acid and arabinose can both be utilized for energy.

Amino Acid Analysis and Comparisons. Because amino acids were identified as being affected by BRD infection at all sampling times, targeted analysis of amino acids was performed. Amino acid data is displayed in Tables 4 through 8, as well as in Appendix 1. At 72 h prior to infection, serine was observed to be lower ($P = 0.042$) in BVDVMH cattle than other treatments, which indicates that the BVDVMH group may have had naturally lower levels of serine (Table 4). There were no differences among treatment groups for total essential, non-essential, glucogenic, and ketogenic amino acids.

At 12 h after infection, changes were observed in glycine and isoleucine (Table 5). Glycine levels were decreased ($P = 0.020$) in BVDVMH steers compared with CTRL and BVDV. Glycine has been shown to be decreased in both pigs dosed with complete Freud's adjuvant, which contains killed *Mycoplasma tuberculosis* cells (Melchior et al., 2004), as well as in cattle dosed with LPS (Waggoner et al., 2009b). Although no changes were apparent in glycine at 12 h after infection in the metabolomic analysis, lower glycine levels were apparent in MH steers at 24 h after infection. Glycine has been implicated as a potential inhibitor of the inflammatory response (Gunderson et al., 2005),

and reduced levels may coincide with an increase in inflammatory substances. Glycine is also a component of glutathione (GSH), which can scavenge free radicals (Wu et al., 2004). Isoleucine was decreased ($P = 0.015$) in both BVDVMH and MH steers compared with CTRL and BVDV steers. Isoleucine was shown to be decreased as well in the metabolomic data for BVDVMH and MH steers. The amino acid results for isoleucine match those of the metabolomic analysis, in which isoleucine levels decreased in both BVDVMH and MH steers.

Changes in amino acid profiles of infected steers were also seen at 24 h after infection (Table 6). Interestingly, leucine, another important BCAA, was higher ($P = 0.001$) in BVDV steers as compared to all other treatment groups. Methionine was observed to have changed similarly, with BVDV steers having increased ($P = 0.008$) plasma concentrations compared to other treatment groups. Isoleucine, threonine, proline, glutamic acid, ornithine, and tryptophan all followed a similar trend where both BVDVMH and MH steers had decreased ($P \leq 0.008$) concentrations of those amino acids, compared with CTRL and BVDV steers. Tryptophan has been implicated as having important function in the immune response which is explained in more detail below. Glutamic acid (glutamate) is another component of glutathione, which can scavenge free radicals (Wu et al., 2004). BVDVMH and MH cattle also had decreased ($P \leq 0.041$) levels of serine and lysine compared with BVDV steers. This may be because of the bacterial infection factor – the faster action of LPS infection causes increased demand of many amino acids for the production of immune system products.

Concentrations of total amino acid classes were different among treatment groups at 24 h after infection. Total essential amino acids were higher ($P = 0.007$) in BVDV

steers than remaining treatment groups. BVDVMH and MH steers had decreased levels of total non-essential ($P = 0.021$) and glucogenic ($P = 0.000$) amino acids compared with CTRL and BVDV steers. Total ketogenic amino acids were greater ($P = 0.001$) in BVDV steers than other groups, and MH steers had the lowest total ketogenic amino acid levels of all treatment groups. These trends follow throughout the data, where steers challenged with MH had lower levels of amino acids than the non-MH challenged groups.

At 48 h after infection, the only changes were seen with methionine and 4-hydroxyproline (Table 7). Methionine and 4-hydroxyproline levels were decreased ($P \leq 0.022$) in BVDVMH and MH steers compared with CTRL. Methionine levels were lower in BVDVMH and MH steers than CTRL steers at both 24 and 48 hours after infection. This is again indicative of the possible faster effect of bacterial infection.

There were also changes in amino acids that were independent of time, i.e., differences among treatments across the entire experiment (Table 7). Serine levels were decreased ($P = 0.022$) in BVDVMH compared with CTRL steers. BVDVMH and MH steers also had lower ($P = 0.022$) serine levels than BVDV steers. The general trends follow those seen in other parts of the analysis, with MH and BVDVMH steers differing from the CTRL and BVDV steers. Two interesting amino acids that decreased ($P \leq 0.041$) were glutamine and tryptophan. Glutamine has been indicated as an important amino acid during the immune response. Specifically, glutamine has been shown to be important for cell growth and as a substrate to enhance growth of immunological cells (Wilmore et al., 1998). Low levels of glutamine, as was seen in BVDVMH and MH cattle when compared to CTRL cattle, have also been shown to decrease major

histocompatibility complex protein presentation by 40% (Juretic et al., 1994). Major histocompatibilty complexes (MHC) are molecules on the surface of cells that present proteins and antigens that can be recognized by T cells. Once T cells contact a MHC that is presenting antigens, the T cell can then proliferate into additional T cells that can recognize and destroy cells that are presenting that antigen (Kindt et al., 2006). A decrease in the levels of glutamine would decrease the ability of the immune system to activate its cells and effectively fight off infection.

Both BVDVMH and MH cattle had lower (P = 0.001) levels of tryptophan than BVDV and CTRL steers. Tryptophan has been shown to decrease with lung inflammation, indicating increased tryptophan catabolism (Melchior et al., 2004). Tryptophan and its metabolites have also been identified as being able to perform functions such as scavenging free radicals (Goda et al., 1999). The decreased levels of tryptophan may be due to the increased utilization of tryptophan to aid in the immune response and ROS scavenging.

Although the metabolomic and amino acid data did not match exactly, there were similarities in the directional changes of amino acids. In the majority of amino acids, even if the changes were not statistically significant, bacterial challenge caused decreases in amino acid concentrations. This was seen in both metabolomic and the targeted analysis data. In addition, the volume of metabolites that were present in the plasma during metabolomic analysis may have inhibited the detection of changes in some less abundant amino acids. In future analyses, separating plasma into fractions based on polarity may allow for the identification of additional metabolites and amino acids that may be relevant biomarkers. The use of metabolomics to identify BRD biomarkers has

promise, although more research is needed in order to confirm results and definitively indentify biomarkers.

Implications

These results indicate that BRD affects both amino acid and energy metabolites. The results identified that amino acids may serve as biomarkers of BRD infection, as well as other metabolites, such as threonic acid. The amino acid targeted analysis did not, however, match all the amino acids observed to be changing during the metabolomic analysis, indicating that further work is needed to confirm biomarker identities. In addition, the establishment of baseline levels of biomarkers will need to be established. It may be beneficial to identify multiple metabolites that would, together, serve as biomarkers of BRD infection. These results indicate it may be possible to identify biomarkers of BRD with further research.

Table 1. Normalized abundances for metabolites found to be significantly changing between control (CTRL) and bovine viral diarrhea virus and *M. haemolytica* infection (BVDVMH), bovine viral diarrhea virus (BVDV), and *M. haemolytica* (MH) treatments at 12 hours after infection (N=24).

Metabolite	CTRL	BVDVMH	BVDV	MH	*P*-value
Isoleucine	1.376	0.220*	1.415	0.468*	0.003
L-glutamic Acid	1.527	0.232*	0.536*	0.181*	0.003
Tyrosine	1.379	0.672	0.910	0.537*	0.005
Threonic Acid	0.103	0.991*	0.610*	0.885*	0.04
Citric Acid	2.748	0.109*	1.114*	0.052*	0.003
Mannitol	0.924	0.190*	0.710	0.075*	0.014
Galacturonic Acid	1.160	0.179*	0.228*	0.395*	0.049
Galactofuranoside	1.084	0.101*	0.336*	0.327*	0.024
Propanoic Acid	1.018	0.297*	0.171*	0.450	0.049
Galactose	0.965	0.425	0.299*	0.160*	0.037

*denotes difference from CTRL steers ($P < 0.05$)

Table 2. Normalized abundances for metabolites found to be significantly changing between control (CTRL) and bovine viral diarrhea virus and *M. haemolytica* infection (BVDVMH), bovine viral diarrhea virus (BVDV), and *M. haemolytica* (MH) treatments at 24 hours after infection (N=24).

Metabolite	CTRL	BVDVMH	BVDV	MH	*P* -value
2-ethyl-3-hydroxypropionic Acid	1.990	0.163*	0.163*	1.523	0.003
Tyrosine	0.872	0.666	1.531	0.337*	0.039
Glycine	0.503	0.971	0.472	0.080*	0.035

*denotes difference from CTRL steers ($P < 0.05$)

Table 3. Normalized abundances for metabolites found to be significantly changing between control (CTRL) and bovine viral diarrhea virus and *M. haemolytica* infection (BVDVMH), bovine viral diarrhea virus (BVDV), and *M. haemolytica* (MH) treatments at 48 hours after infection (N=24).

Metabolite	CTRL	BVDVMH	BVDV	MH	*P*-value
Alanine	0.214	1.111*	0.936*	0.457	0.0139
Glycine	1.248	0.095*	0.269*	0.665*	0.020
Phenylalanine	0.191	0.033*	1.409*	0.125*	0.006
Valine	0.462	0.073*	1.236*	0.323	<0.01
Leucine	0.230	0.072*	1.191*	0.099	<0.01
Arabinose	0.400	0.627	1.190*	0.571	0.024
Acetic Acid	0.989	0.951	0.110*	0.214*	0.040

*denotes difference from CTRL steers ($P < 0.05$)

Table 4. Least square means of plasma amino acid concentrations (nM/mL) of steers infected with either bovine viral diarrhea virus (BVDV) and/or *M. haemolytica* (MH) at 72 h prior to infection (N=24).

Amino Acid	CTRL	BVDVMH	BVDV	MH	SE	*P*-value
Valine	207.72	337.66	441.84	245.28	42.68	0.165
Leucine	112.92	186.69	152.74	157.83	12.39	0.318
Isoleucine	74.30	85.04	84.72	82.09	2.04	0.891
Threonine	38.87	40.24	40.76	33.56	1.35	0.867
Methionine	5.66	6.55	6.09	6.64	0.18	0.983
Phenylalanine	40.03	51.48	48.76	49.04	2.05	0.563
Lysine	71.81	61.16	80.14	69.03	3.20	0.999
Histidine	4.15	3.52	4.62	3.57	0.21	0.679
Tryptophan	16.51	16.35	21.74	15.19	1.19	0.402
Alanine	365.32	368.97	388.56	377.51	4.22	0.980
Sarcosine	2.74	0.00	4.01	0.00	0.82	0.520
Glycine	276.20	259.85	301.59	283.11	7.06	0.960
α-Aminobutyric Acid	7.60	13.96	10.45	8.61	1.14	0.437
Serine	104.13^{b}	56.53^{a}	118.49^{b}	101.62^{b}	3.72	0.042
Proline	93.86	96.88	99.12	101.09	1.27	0.973
Asparagine	13.02	10.86	18.36	17.37	1.45	0.813
Aspartic Acid	2.80	1.75	2.09	2.52	0.19	0.797
4-Hydroxyproline	20.95	18.45	23.77	19.77	0.93	0.730
Glutamic Acid	370.39	361.43	396.96	397.72	7.56	0.961
Glutamine	56.64	26.07	39.98	41.07	5.10	0.743
Ornithine	71.61	101.57	103.15	114.76	7.51	0.705
Tyrosine	23.88	33.46	28.41	32.10	1.75	0.829
Essential AA	562.81	797.88	876.76	662.23	57.05	0.145
Non-Essential AA	1373.73	1382.27	1517.04	1496.77	30.60	0.893
Glucogenic AA	1791.00	1832.15	2153.82	1903.76	66.37	0.580
Ketogenic AA	374.79	477.95	455.86	438.84	18.11	0.676

[a] within row, means with different superscripts differ ($P < 0.05$).

Table 5. Least square means of plasma amino acid concentrations (nM/mL) of steers infected with either bovine viral diarrhea virus (BVDV) and/or *M. haemolytica* (MH) at 12 h after infection (N=24).

Amino Acid	CTRL	BVDVMH	BVDV	MH	SE	*P*-value
Valine	340.69	387.84	419.60	242.54	31.52	0.562
Leucine	123.64	99.82	157.70	77.29	14.04	0.229
Isoleucine	76.34[a]	32.41[b]	77.24[a]	33.09[b]	10.38	0.015
Threonine	31.57	20.93	46.88	15.76	5.61	0.347
Methionine	5.93	2.50	6.04	2.97	0.77	0.203
Phenylalanine	53.95	48.42	60.76	42.08	3.25	0.354
Lysine	49.36	18.67	40.82	23.29	5.91	0.091
Histidine	4.46	6.20	5.84	10.64	1.09	0.080
Tryptophan	17.31	10.71	14.79	9.79	1.44	0.342
Alanine	309.98	296.46	305.05	240.52	13.12	0.362
Sarcosine	4.61	5.08	2.92	3.99	0.38	0.570
Glycine	202.41[b]	112.80[a]	231.39[b]	172.20[ab]	20.71	0.020
α-Aminobutyric Acid	8.69	6.31	8.43	5.60	0.63	0.079
Serine	67.73	44.34	77.01	48.31	6.37	0.503
Proline	81.47	66.14	89.62	55.25	6.28	0.055
Asparagine	6.46	5.62	9.86	5.85	0.81	0.511
Aspartic Acid	1.94	N.D.[*]	3.88	1.63	0.50	0.063
4-Hydroxyproline	20.86	18.87	21.88	17.66	0.78	0.699
Glutamic Acid	282.25	207.43	250.38	172.47	19.65	0.158
Glutamine	75.30	53.89	50.75	58.09	4.47	0.497
Ornithine	45.36	25.79	46.52	32.47	4.12	0.432
Tyrosine	18.89	10.80	22.33	10.27	2.44	0.183
Essential AA	703.25	603.28	820.73	454.12	63.44	0.183
Non-Essential AA	1121.02	843.42	1097.78	820.16	65.66	0.145
Glucogenic AA	1621.73	1291.43	1688.30	1150.05	105.62	0.143
Ketogenic AA	371.07	241.91	422.14	211.57	41.24	0.188

[ab] within row, means with different superscripts differ ($P < 0.05$).

[*] concentration was not high enough to be detected

Table 6. Least square means of plasma amino acid concentrations (nM/mL) of steers infected with either bovine viral diarrhea virus (BVDV) and/or *M. haemolytica* (MH) at 24 h after infection (N=24).

Amino Acid	CTRL	BVDVMH	BVDV	MH	SE	*P*-value
Valine	313.10	279.55	368.47	319.25	14.97	0.628
Leucine	115.14[a]	124.92[a]	183.13[b]	94.30[a]	15.53	0.001
Isoleucine	71.47[a]	46.98[b]	91.78[c]	47.08[b]	8.83	0.000
Threonine	30.80[b]	16.22[a]	53.80[b]	12.84[a]	7.61	0.000
Methionine	5.67[a]	4.43[a]	8.27[b]	4.02[a]	0.78	0.008
Phenylalanine	52.10	72.71	70.06	60.40	3.85	0.383
Lysine	51.55[ab]	32.37[c]	61.65[a]	35.20[bc]	5.65	0.041
Histidine	6.03	7.82	6.15	9.54	0.67	0.584
Tryptophan	18.58[a]	9.34[b]	19.44[a]	8.83[b]	2.35	0.001
Alanine	337.67	260.42	296.58	276.87	13.60	0.436
Sarcosine	3.30	3.83	2.88	2.62	0.22	0.786
Glycine	242.60	100.88	187.81	136.41	25.23	0.061
α-Aminobutyric Acid	9.56	6.96	7.15	6.56	0.55	0.431
Serine	69.24[ab]	49.44[b]	89.78[a]	45.04[b]	8.37	0.014
Proline	79.39[a]	64.87[b]	86.25[a]	58.55[b]	5.21	0.000
Asparagine	10.37	7.07	8.86	4.98	0.95	0.292
Aspartic Acid	6.70	1.58	3.24	1.77	0.97	0.071
4-Hydroxyproline	22.89	16.24	24.33	16.16	1.76	0.210
Glutamic Acid	325.15[a]	217.66[b]	279.77[a]	202.75[b]	23.16	0.00
Glutamine	78.93	34.39	69.77	46.42	8.39	0.225
Ornithine	57.24[a]	36.05[b]	65.94[a]	35.68[b]	6.23	0.001
Tyrosine	17.44	20.73	24.28	14.99	1.65	0.341
Essential AA	664.43[a]	595.86[a]	862.75[b]	590.42[a]	52.03	0.007
Non-Essential AA	1255.10[a]	817.52[b]	1143.20[a]	845.30[b]	88.78	0.021
Glucogenic AA	1721.00[a]	1227.62[b]	1729.68[a]	1283.50[b]	111.13	0.00
Ketogenic AA	357.07[a]	323.93[a]	504.14[b]	273.63[c]	40.45	0.001

[abc] within row, means with different superscripts differ ($P < 0.05$).

Table 7. Least square means of plasma amino acid concentrations (nM/mL) of steers infected with either bovine viral diarrhea virus (BVDV) and/or *M. haemolytica* (MH) at 48 h after infection (TP+48; N=24).

Amino Acid	CTRL	BVDVMH	BVDV	MH	SE	*P*-value
Valine	354.21	376.93	408.88	431.21	13.89	0.921
Leucine	142.97	167.37	177.98	119.91	10.60	0.657
Isoleucine	85.54	78.23	95.36	72.34	4.05	0.644
Threonine	44.88[a]	25.00[b]	52.23[a]	23.87[b]	5.82	0.005
Methionine	6.91[a]	3.54[bc]	6.00[ab]	3.24[c]	0.74	0.022
Phenylalanine	60.61	54.51	65.33	48.80	2.94	0.647
Lysine	61.56	59.52	55.39	46.82	2.66	0.683
Histidine	7.77	8.91	7.10	7.39	0.32	0.843
Tryptophan	21.75	12.33	16.04	12.16	1.83	0.971
Alanine	329.28	207.26	267.30	223.03	22.31	0.126
Sarcosine	5.37	4.34	3.49	4.25	0.32	0.768
Glycine	216.31	105.87	165.17	162.35	18.42	0.055
α-Aminobutyric Acid	7.46	7.00	6.45	5.26	0.39	0.754
Serine	77.09	48.08	78.26	55.00	6.27	0.142
Proline	80.24	71.85	77.72	63.44	3.05	0.555
Asparagine	10.11	5.74	9.12	6.08	0.89	0.411
Aspartic Acid	3.78	4.52	3.48	3.56	0.19	0.886
4-Hydroxyproline	23.73[a]	16.87[b]	20.81[ab]	15.67[b]	1.51	0.013
Glutamic Acid	270.81	202.31	242.19	213.70	12.53	0.067
Glutamine	80.69	47.99	57.79	45.26	6.57	0.165
Ornithine	65.82	64.58	59.15	42.18	4.45	0.650
Tyrosine	23.87	12.62	23.18	13.11	2.52	0.066
Essential AA	786.20	811.81	885.95	765.44	21.48	0.931
Non-Essential AA	1189.18	799.86	1010.26	856.65	71.22	0.355
Glucogenic AA	1739.66	1413.91	1625.38	1423.98	65.00	0.490
Ketogenic AA	441.18	407.47	485.00	335.37	25.78	0.528

[abc] within row, means with different superscripts differ ($P < 0.05$).

Table 8. Least square means of plasma concentrations (µM/mL) for amino acids that were affected overall by bovine viral diarrhea virus (BVDV) and/or *M. haemolytica* (MH) infection over total experiment time (N=24).

Amino Acid	CTRL	BVDVMH	BVDV	MH	SE	*P*- value
Serine	79.24^{ab}	52.53^{c}	88.36^{a}	62.54^{bc}	6.59	0.022
Proline	83.76^{ab}	74.15^{ac}	87.95^{b}	69.85^{c}	3.42	0.029
Glutamine	74.67^{a}	38.34^{b}	55.88^{ab}	48.07^{b}	6.28	0.041
Tryptophan	18.83^{a}	12.11^{b}	17.83^{a}	11.43^{b}	1.56	0.001

^{abc} within row, means with different superscripts differ ($P < 0.05$).

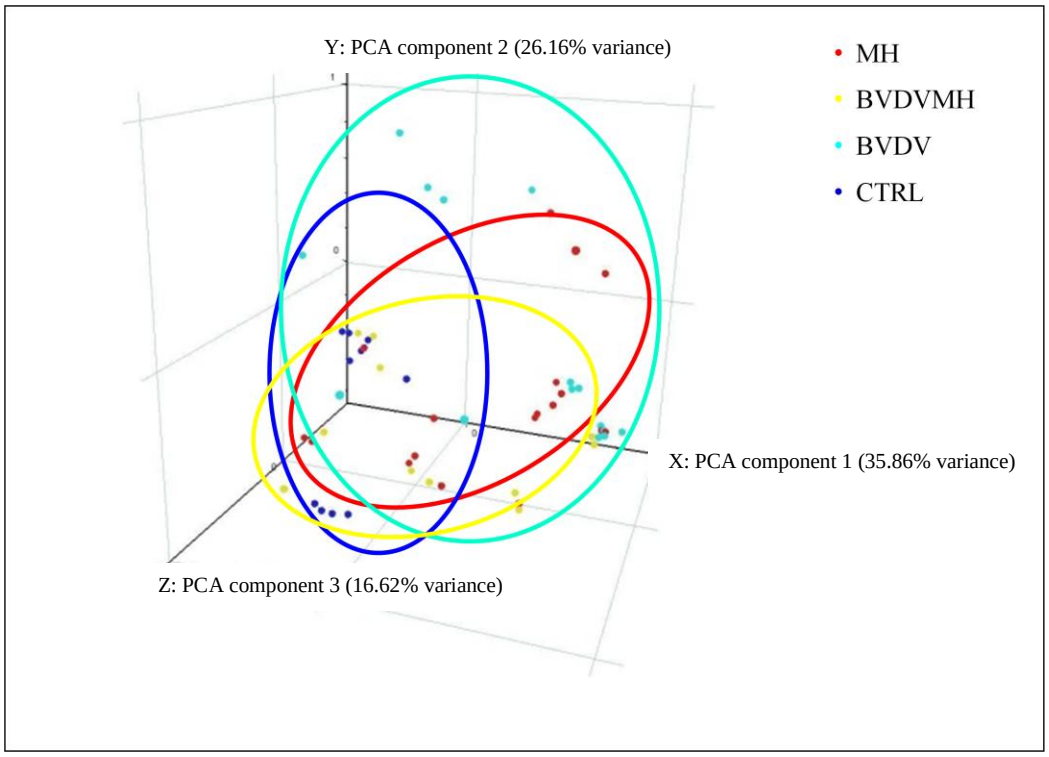

Figure 1. Principal component analysis of 72 hours prior to infection (TP-72; N=24). Treatments are indicated by the colors shown in legend. Circles are color coded in accordance to the treatment they encircle.

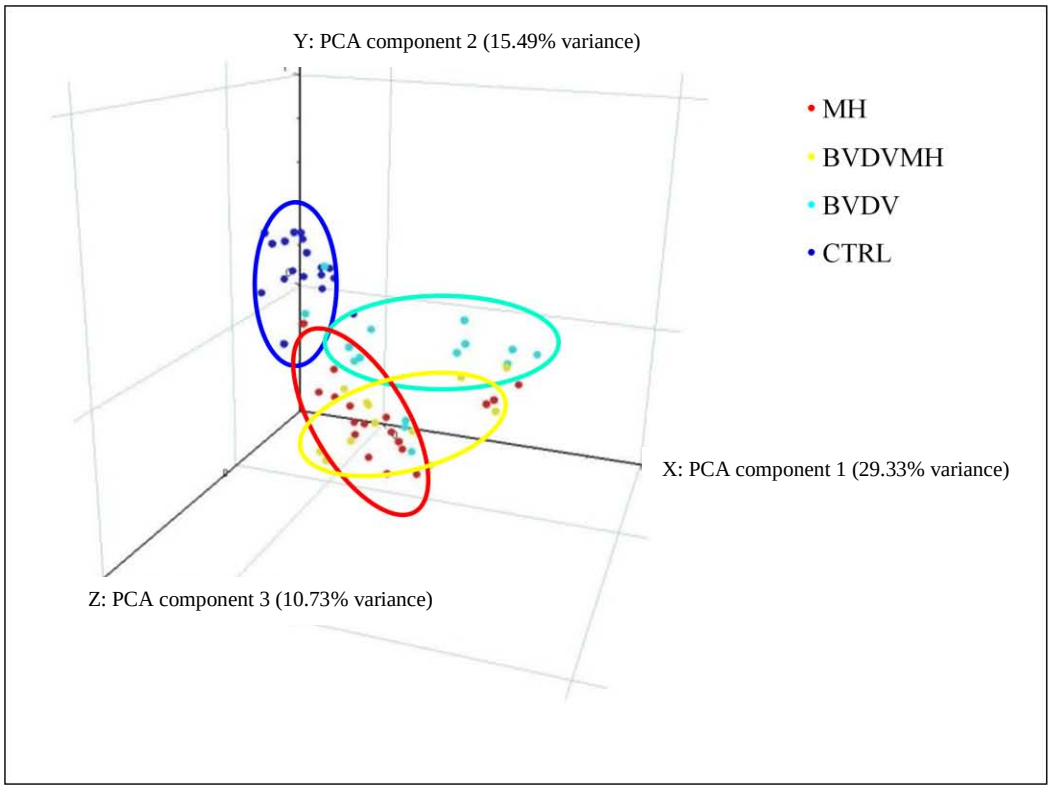

Figure 2. Principal component analysis of 12 hours after infection (TP+12; N=24). Treatments are indicated by the colors shown in legend. Circles are color coded in accordance to the treatment they encircle.

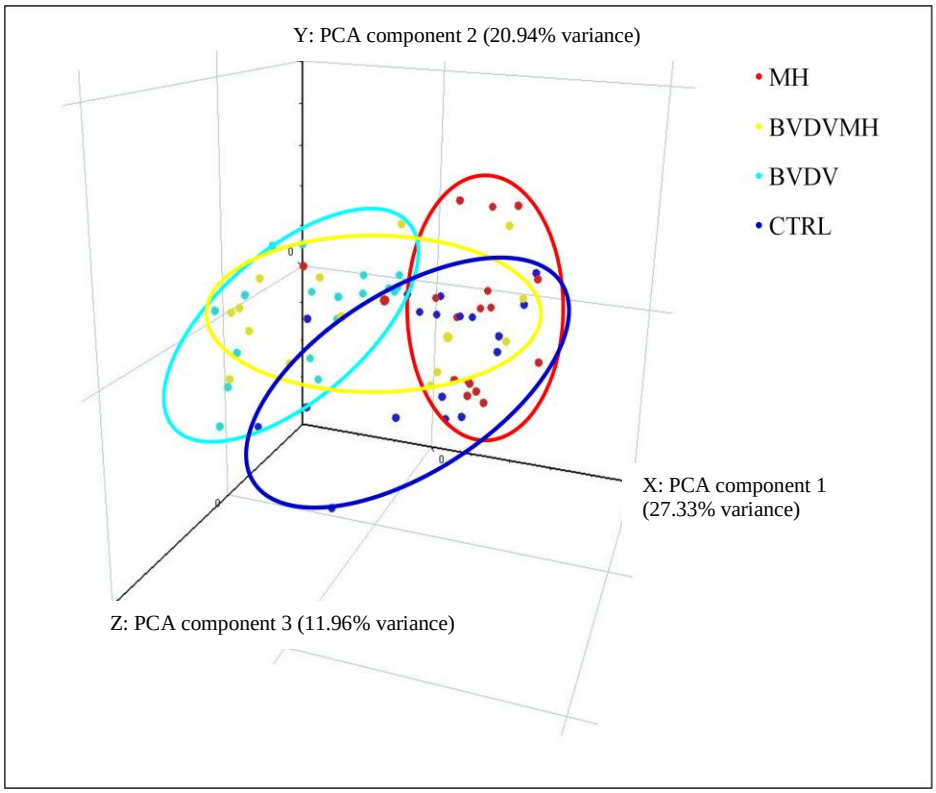

Figure 3. Principal component analysis of 24 hours after infection (TP+24; N=24). Treatments are indicated by the colors shown in legend. Circles are color coded in accordance to the treatment they encircle.

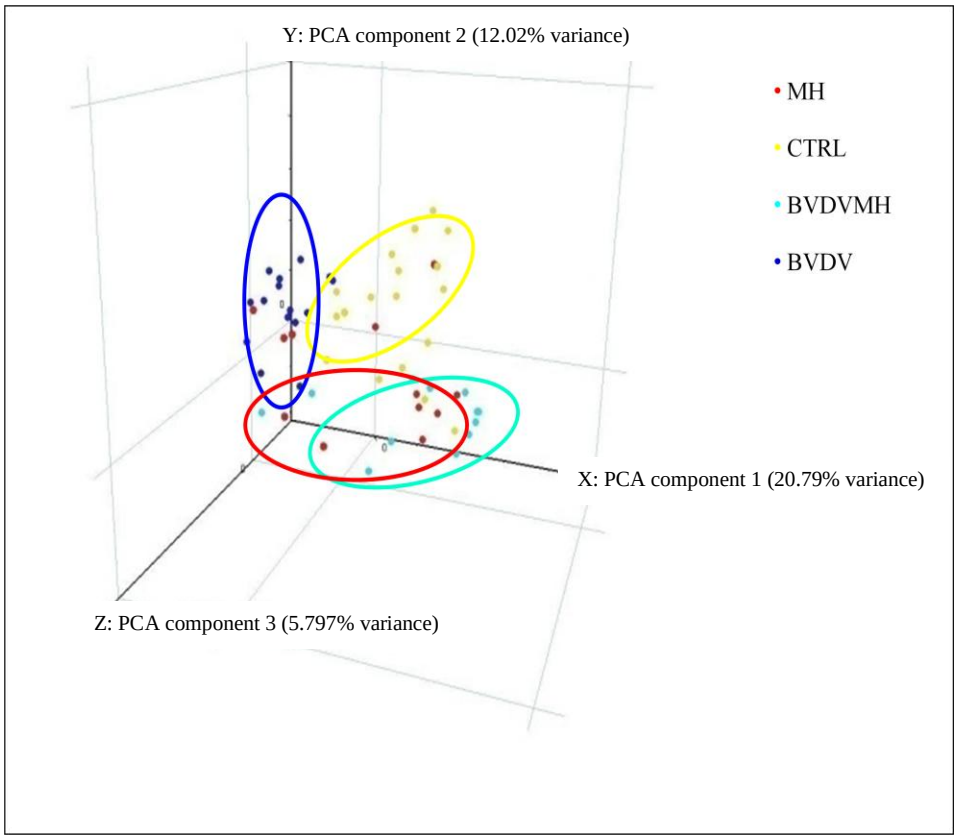

Figure 4. Principal component analysis of 48 hours after infection (TP+48; N=24). Treatments are indicated by the colors shown in legend. Circles are color coded in accordance to the treatment they encircle.

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APPENDICES

Appendix 1. Least square means of all concentrations of plasma amino acids for bovine viral diarrhea virus (BVDV) and/or M. haemolytica (MH) challenged steers across all time points (N=24). Differences among all treatments are displayed.

	TP-72					TP+12					TP+24					TP+48					
Amino Acid	CTRL	BVDVMH	BVDV	MH	SE	CTRL	BVDVMH	BVDV	MH	SE	CTRL	BVDVMH	BVDV	MH	SE	CTRL	BVDVMH	BVDV	MH	SE	*P* -value
Alanine	357.37	374.09	387.43	377.51	19.58	309.98	288.17	306.12	240.52	19.10	337.66	262.22	296.58	276.87	18.38	329.28	209.83	265.56	232.89	19.73	0.367
Sarcosine	4.56	N.D.*	4.01	N.D.*	N.D.*	4.61	5.23	2.98	3.98	0.54	3.37	3.83	2.88	2.62	0.48	5.22	4.09	3.61	4.26	0.47	0.883
Glycine	277.10	264.38	299.66	283.11	39.88	202.41	111.90	216.20	172.20	39.23	242.26	97.61	187.81	136.41	39.11	216.31	128.77	165.85	178.32	39.56	0.083
α-Aminobutyric Acid	6.16	15.35	9.77	8.61	1.83	8.69	6.06	8.07	6.00	1.79	9.55	6.62	7.15	6.56	1.78	7.46	6.40	6.28	6.10	1.81	0.208
Valine	248.63	317.22	454.61	245.28	40.14	340.69	392.94	428.40	242.54	38.53	313.10	286.46	368.47	319.25	36.39	354.21	355.47	405.81	429.75	40.55	0.780
Leucine	124.24	183.48	156.68	157.83	17.87	123.64	108.94	152.87	77.29	17.44	115.14	129.09	183.13	94.30	17.26	142.97	151.86	177.51	128.53	17.76	0.159
Isoleucine	80.36	83.81	85.90	82.09	4.51	76.34^{a}	35.77^{b}	77.46^{a}	33.09^{b}	4.28	71.47^{ab}	47.73^{ac}	91.78^{b}	47.08^{c}	4.09	76.34	35.77	77.46	33.09	4.54	0.021
Threonine	41.51	37.39	42.02	33.56	8.04	31.57^{ab}	24.38^{ab}	41.99^{a}	15.76^{b}	7.95	30.83^{a}	17.4^{ab}	53.8^{c}	12.84^{bc}	7.92	44.88^{a}	26.37^{b}	52.11^{a}	21.78^{b}	8.01	0.031
Serine	102.91	57.59	119.48	101.63	6.04	67.73	48.08	65.10	48.31	5.84	69.24	49.85	89.78	45.04	5.53	77.09	54.60	79.08	55.19	6.21	0.266
Proline	93.92	97.02	99.11	101.09	3.79	81.47	64.48	87.80	55.25	3.57	79.39	64.87	86.25	58.55	3.38	80.24	70.21	78.64	64.51	3.83	0.478
Asparagine	12.75	10.91	18.47	17.37	2.03	6.46	5.77	8.92	5.85	1.85	10.37	7.18	8.86	4.98	1.81	10.11	6.08	9.78	5.37	1.96	0.912
Aspartic Acid	2.89	1.68	2.09	2.49	2.29	1.90	N.D.*	3.81	1.63	N.D.*	6.77^{b}	1.46^{a}	3.3^{a}	1.88^{a}	0.60	3.78	4.16	3.54	3.68	0.59	0.027
Methionine	6.01	6.42	6.35	6.64	1.13	5.92	2.86	5.88	2.92	1.12	5.67	4.38	8.27	4.02	1.10	6.90	4.16	6.00	3.52	1.14	0.304
4-Hydroxyproline	20.58	17.95	23.82	19.77	1.76	20.86	17.79	20.80	17.66	1.73	22.29	16.66	24.33	16.16	1.68	23.73	17.07	21.44	16.22	1.78	0.835
Glutamic Acid	343.41	388.82	384.37	397.72	34.42	282.25	196.63	246.10	172.47	33.59	202.75	212.64	279.77	202.75	33.22	270.81	200.54	236.21	214.42	34.35	0.667
Phenylalanine	45.19	50.67	50.13	49.04	8.67	53.95	49.35	59.19	42.08	8.64	52.10	73.46	70.06	60.40	8.58	60.61	52.00	65.94	51.80	8.68	0.180
Glutamine	63.74	18.11	44.61	41.07	8.98	75.30	50.48	51.32	58.09	8.93	78.93	35.02	69.77	46.42	8.33	80.69	49.76	57.82	46.71	9.17	0.967
Ornithine	76.87	97.54	104.13	114.76	17.38	45.36	23.27	47.14	32.47	17.05	57.24	36.62	65.94	35.68	16.98	65.82	53.92	57.88	53.90	17.22	0.537
Lysine	71.66	62.30	78.93	69.03	5.95	49.36	17.87	40.08	23.29	5.40	51.55	32.14	61.65	35.20	5.19	61.56	60.65	55.74	48.97	5.68	0.601
Histidine	3.65	4.09	4.62	3.57	1.36	4.46	6.08	6.44	10.40	1.37	6.03	7.83	6.15	9.89	1.33	7.77	9.21	7.14	8.29	1.35	0.230
Tyrosine	25.63	31.90	29.10	32.10	5.33	18.89	11.51	20.95	10.27	5.24	17.44	21.24	24.28	14.99	5.21	23.87	14.83	22.84	14.34	5.31	0.321
Tryptophan	17.67	16.11	21.93	15.19	4.35	17.31	10.62	14.36	9.79	4.35	18.58	9.45	19.44	8.83	4.32	21.75	12.24	15.59	11.93	4.35	0.137
Essential AA	650.57	773.35	902.07	662.23	78.42	703.25^{ab}	657.16^{ab}	829.59^{a}	454.12^{b}	78.14	664.43	613.36	862.75	590.42	75.76	786.20	754.15	883.99	789.67	78.38	0.000
Non-Essential AA	1374.56	1383.63	1516.73	1496.77	52.98	1121.02	823.88	1073.11	820.16	50.86	1255.10	816.73	1143.20	845.30	47.33	1189.18	815.54	1017.11	886.88	54.08	0.436
Glucogenic AA	1805.80	1851.16	2157.71	1903.75	68.70	1621.73	1297.94	1671.64	1150.05	66.11	1721.00	1233.36	1729.68	1283.50	61.81	1739.66	1305.38	1631.11	1469.83	68.70	0.189
Ketogenic AA	410.15	463.70	466.13	351.94	48.98	371.07	255.59	408.60	211.57	48.42	357.07	329.31	504.14	273.63	47.85	441.18	387.11	438.84	484.88	49.06	0.152

^{abc} means across a row within a time point are significantly different ($P < 0.05$).

* concentration was not high enough to be detected

VITA

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Pages in Study: 64 Candidate for the Degree of Master of Science

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Scope and Method of Study: Bovine respiratory disease (BRD) is the most common disease in North American feedlots. The whole metabolome of plasma from BRD infected calves was analyzed using gas chromatography-mass spectrometry for changing metabolites as compared to uninfected cattle to determine metabolites that could be used as biomarkers of disease. Targeted analysis was then performed on amino acids also using gas chromatography coupled with mass spectrometry.

Findings and Conclusions: There were significant changes observed in the plasma of infected cattle. The compounds included both amino acids and energy substrates. Targeted analysis of amino acid concentrations in plasma had similar directional changes as the metabolomic analysis, although there were some differences in the amino acids that changed. Examining the whole metabolome may be a beneficial way to identify potential biomarkers of BRD, however further research is needed to validate and confirm biomarker identities.

ADVISER'S APPROVAL: _____

Dr. Clint Krehbiel