THE INNATE IMMUNE RESPONSE TO SUBACUTE RUMINAL ACIDOSIS IN BEEF CATTLE

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Title of Study: THE INNATE IMMUNE RESPONSE TO SUBACUTE RUMINAL ACIDOSIS IN BEEF CATTLE

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Abstract: Background: Subacute ruminal acidosis (SARA) is a metabolic disease that affects both dairy and beef cattle. The effects of SARA have been extensively studied in dairy cattle, but information in beef cattle is limited. Grain-induced subacute ruminal acidosis is associated with systemic inflammation in both beef and dairy cattle. The insidious nature of SARA, coupled with lack of specific signs means that the condition is quite difficult to diagnose in beef cattle.

Hypotheses: SARA occurs naturally in beef cattle that are undergoing preconditioning at a backgrounding facility. SARA causes ruminal bacteria population changes coupled with increase in levels of free endotoxin and biogenic amines in the rumen. The ruminal changes are associated with increased levels of endotoxin and increased expression of inflammatory cytokine in broncho-alveolar lavage (BALF) and blood.

Materials and Methods: Rumenocentesis was performed to evaluate the occurrence of SARA in cattle being fed preconditioning diets. Ruminal fluid pH was also measured in animals that were identified as having bovine respiratory disease. Ruminal bacteria population changes were evaluated using qRT-PCR. Endotoxin concentration in ruminal fluid and plasma was quantified using a kinetic chromogenic Limulus Amoebocyte Lysate test. The expression inflammatory cytokines in BALF and blood was assessed with qRT-PCR. An HPLC method was developed to quantify biogenic amines in ruminal fluid.

Results: Rumenocentesis was performed without complications. Thirteen out of sixty (22%) cattle had ruminal pH $\leq$5.6 on day 21 in one the studies. Lactate-utilizing Gram negative bacteria, *Megasphaera elsdenii* was significantly up-regulated in groups of animals being fed high amounts of fermentable carbohydrates. Ruminal fluid and plasma endotoxin concentration increased with time in animals being fed varying amounts of highly fermentable carbohydrates. The concentration of endotoxin was correlated with the quantity of highly fermentable carbohydrates in the diet. The concentration of biogenic amines was higher in animals receiving higher amounts of highly fermentable carbohydrates. Pro-inflammatory cytokine were upregulated in both ruminal fluid and blood for the first two weeks. On the third week, IL-1β and TNF were down-regulated.

Conclusion: The innate immune response to SARA resembles endotoxin tolerance. Endotoxin tolerance needs further investigation in cattle.

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CHAPTER I

SUBACUTE RUMINAL ACIDOSIS- A REVIEW

Introduction

The economics of beef and dairy production dictate that cattle need to gain weight or produce milk at their respective maximum potential rates.[1,2] Feedlot and dairy cattle are therefore, often fed soluble carbohydrate diets to maximize production. Unfortunately, consumption of soluble carbohydrate diets can be associated with increased morbidities and decreased production.[3] An important consideration at the herd level is subacute ruminal acidosis (SARA). Subacute ruminal acidosis is a metabolic disease associated with peri-parturient diseases of dairy cows such as hypocalcemia, ketosis and displacement of the abomasum.[4] Subacute ruminal acidosis has also been associated with bovine respiratory disease (BRD) in beef cattle.[5,6] The mechanisms and the extent of the relationship between SARA and BRD have not been elucidated.

Ruminants and Carbohydrate Digestion

It has been suggested that ruminant digestion has changed over time from predominantly dependent on plant cell hind gut fermentation, to unselective roughage fermentation in the forestomachs.[1] Beef cattle face potential problems because as grass feeders they are adapted to forage diets but the economics of feedlot beef production dictate that cattle should gain weight at their maximum efficient potential rate; this usually involves feeding them a diet containing concentrates with grains.[3] In addition, consumers in some countries including the United States and Canada are more familiar with beef produced from feeding both grain and grass than for beef produced from feeding grass only.[7] Similarly, dairy cattle frequently are fed diets containing highly fermentable carbohydrates to meet their energy demands and to optimize milk production.[2]

There exists a synergistic relationship between the rumen and the ruminal microbes.[8] Fermentation in the rumen is the result of breakdown of feedstuffs through catabolic processes aided by the microorganisms to useful products including volatile fatty acids, microbial protein, B-vitamins and other byproducts such as methane, carbon dioxide, ammonia and nitrite.[8] The microorganisms that reside in the rumen include bacteria, protozoa and anaerobic fungi. These microorganisms depend on the ruminant to provide the physiological conditions necessary for their survival as well as providing a source of nutrients. Likewise, these microorganisms are responsible for the digestion and fermentation of the fibrous feed that ruminants would otherwise be unable to utilize.[9]

Ruminal bacteria can be classified into groups according to shape, size and structure. There are three main shapes, namely, cocci, rods and spirilla with sizes ranging from 0.3 to 50 μm. Their various distinguishing structures include the presence of a cell envelope, cytoplasmic structures and surface adherents or appendages.[10] The bacteria can also be classified according to their utilization of cellulose, hemicelluloses, starch, sugars, intermediate acids, proteins and lipids.[10] In general, major cellulolytic and hemicellulolytic species such as *Rumminococcus* spp and lactate-utilizing species,

Megasphera elsdenii and *Selomonas ruminantium,* are Gram-negative whereas amylotic, lactate-producing species such as *Streptococcus bovis* and *Lactobacilus* spp and are Gram-positive.[10]

Bacteria have different preferences and affinities for various substrates. Carbohydrate fermentation efficiency, which is dependent upon the amount of ATP produced per each carbohydrate molecule digested, provides competitive survival value.[11] Cellulose and hemicellulose utilizers are slow growing but more efficient in ATP utilization than amylolytic bacteria. They are thus at competitive advantage in the ruminal environment.[11] In normal situations, there are more Gram-negative bacteria in the rumen to digest the cellulose and hemicellulose feed that make up the bulk of the ruminant diet. However, when starch is available in excess, the fast growing amylolytic bacteria metabolize carbohydrates faster and produce more ATP per unit time.

Ruminal acidosis syndromes

Definitions

Ruminal acidosis is a metabolic syndrome of ruminants that occurs following ingestion of highly fermentable carbohydrates.[12] There are two forms of the condition; a life-threatening acute disease, ruminal lactic acidosis (RLA) and an insidious condition with less obvious clinical signs, subacute ruminal acidosis (SARA).[13,14,15] Ruminal lactic acidosis is characterized by ruminal fluid pH values less than 5.2 whereas SARA is characterized by a ruminal fluid pH range of 5.2-5.5 for at least 3 hours per day.[16,17,18] Elam outlined scenarios that predispose feedlot cattle to RLA; including weather changes, breakdown of equipment, personnel errors, introduction to concentrate feed, short adaptation period and long-term feeding of high concentrate diets.[19] RLA can be induced by a wide variety of traditional feedstuffs including wheat, barley, rye, oats and corn.[12] Feeding other non-traditional feedstuffs such as sugar beets and potatoes can also result in ruminal acidosis.[20]

Ruminal bacteria changes

Until recently, the microbial changes in SARA were not known.[21,22] Microbial changes in SARA resemble those that occur during adaptation to grain feeding.[21] Total viable anaerobic and amylolytic bacterial counts were shown to be increased in experimentally induced SARA in steers.[21] Lactate-utilizing bacterial counts were also increased in that study. Although limited, recent molecular studies appear to support the earlier culture based experiments that demonstrated decreases in cellulolytic bacteria such as *Ruminococcus* spp to lactate-utilizing bacteria such as *Megasphaera elsdenii* and *Selemonas ruminantium* when cattle were transitioned from forage based diets to grain diets.[23,24,25]

In contrast, microbial changes in RLA have been extensively studied. [9,19,21] In the normal anaerobic ruminal environment of grass fed cattle, the pH is above 6. 5 and there is a microbial population of protozoa and predominant gram-negative bacteria.[9] Consumption of excessive amounts of fermentable carbohydrates results in increased fermentation and volatile fatty acids (VFA) production, resulting in moderately acidic ruminal fluid pH with values ranging from 5 to 5.5.[21] The ruminal fluid pH further decreases to levels below 5.0 because of the production of lactic acid by *Streptococcus bovis* .[21] At this pH, most Gram-negative, lactate- utilizing bacteria including *Megasphaera elsdenii* and *Selomonas ruminantium* die off.[26] The death of other substrate-competing bacteria and the increasing acidity of the fluid results in proliferation of another lactate producing bacteria, *Lactobacillus* spp.[26] Even the *S. bovis* that began the lactic acid production is inhibited below pH 4.5, leaving *Lactobacillus* spp, the most acid-resistant species, to generate more lactic acid .[26]

Rumen physiology changes

Rumen microbial fermentation converts carbohydrates to volatile fatty acids, lactate, carbon dioxide and methane.[19] Consumption of excessive amounts of fermentable carbohydrates results in increased fermentation and volatile fatty acids (VFA) production. Both the increasing concentrations of VFAs and accumulating lactate are responsible for the increase in ruminal osmolality.[26] In a normal animal,

ruminal osmolality is maintained at approximately 280 mOsm/L, but the value may double in some cases of RLA.[26] The increased osmolality draws fluid from the extra-cellular space into the rumen resulting in increased ruminal fluid volume.[26] Lactate is not readily absorbed into circulation because of the hypertonicity of the ruminal fluid, but the detection of high levels of D- lactate in circulation provides evidence that significant absorption does occur.[26] Ruminal acidosis also causes an increase in the concentration of endotoxin in the ruminal fluid.[27] Other substances such as biogenic amines and ethanol also increase in the ruminal fluid.[28]

Although lactate plays a significant role in the pathogenesis of acute ruminal acidosis, its levels are generally not increased in SARA because of the concomitant increase in lactate utilizers.[22] Therefore, the decrease in ruminal fluid pH observed in acute ruminal acidosis is a combination of increased VFA concentrations and lactate accumulation, whereas the decrease in pH observed in SARA is a result of increased VFA concentration only.[29] There is a shift in the proportion of volatile fatty acids in the ruminal fluid of cattle with SARA. The molar proportions of butyrate and propionate increase while acetate decreases in cases of SARA.[29,30,31] Increasing proportions of butyrate and propionate by the ruminal bacteria stimulate proliferation of rumen papillae.[11] An exaggeration of this process results in parakeratosis of the rumen papillae, a situation that predisposes it to trauma and also prevents absorption of volatile fatty acids resulting in further decreases in pH.[11]

Pathologic and systemic physiologic changes

Cattle with experimentally induced ruminal acidosis have elevated blood arachidonic acid, but the role of this molecule in naturally occurring disease is unclear.[32,33] The low ruminal pH may favor the proliferation of fungus resulting in mycotic rumenitis.[20] Ruminal acidosis also causes proliferation of thiaminase producing bacteria and H_2S producing bacteria, and death of thiamine producing bacteria.[26] Thiamine deficiency and H_2S toxicity lead to neuronal edema, likely because of the

disturbances of ATP production pathways.[34,35,36,37] Laminitis, the other sequela of ruminal acidosis, is covered under the 'effects of SARA on animal welfare' subtitle.

Lactic acid, produced in acute ruminal acidosis, is corrosive to the ruminal mucosa and can result in a toxic rumenitis.[29] Feeding soluble carbohydrates has been associated with parakeratosis and hyperkeratosis of the rumen wall.[38] In addition, ruminal acidosis and SARA have been associated with other structural and molecular changes to the stratified squamous epithelium.[38] The damaged mucosa facilitates translocation of bacteria and endotoxin into the systemic circulation.[29] The various mechanism of these changes, including possible interactions with ruminal microbes, have been extensively studied and reviewed.[38] The translocated bacteria can be seeded in other body organs including the liver where they cause liver abscesses.[39] The liver abscesses may erode through the caudal vena cava resulting in formation of thrombus.[39,40,41] Septic emboli detach from the thrombus and reach the lungs through the pulmonary arterial system.[41] Smaller emboli lodge in the arterioles where they cause thromboembolism, arteritis, endarteritis, and pulmonary abscesses.[40] Arteritis and endarteritis in combination with pulmonary hypertension result in the formation of aneurysms. In some cases, a perivascular abscess may not only erode an arterial wall to produce an aneurysm, but also erodes a bronchial wall and when the aneurysm ruptures, the abscess cavity channels the blood into the bronchus, resulting in massive hemoptysis.[40] Occasionally, large emboli may block lobar or larger arteries, causing an acute crisis and death.[40] The entire syndrome is collectively called caudal vena caval thrombosis, pulmonary thromboembolism, embolic pulmonary aneurysm and hemoptysis or simply caudal vena caval thrombosis syndrome.[40,42]

The liver responds to grain-induced SARA by producing acute phase proteins that can modify immune function and generate a systemic inflammatory response. The main bovine acute phase proteins are serum amyloid A, haptoglobin, LPS-binding protein, and α-1 acid glycoprotein.[38] Acute phase proteins stimulate tissue repair, remove harmful compounds, isolate infectious agents, and

prevent further damage.[38] Although a lot is known about acute phase proteins, little is known about the direct interaction of the immune system with compounds that are translocated from the rumen.

Clinical signs

Acute ruminal acidosis affects ruminants of any age, breed or sex.[43] Morbidity within a herd can range from 2% to 50% while mortality can range from 30% to 40% in treated animals and can be as high as 90% in untreated animals.[43] Clinical signs of acute ruminal acidosis depend on the type and amount of feed consumed, and the time at which the animal is examined relative to the time of consumption.[44] Endotoxin is considered to be a major factor in the development of clinical signs but this assertion has not been entirely proven.[44] Initially, cattle are thirsty and have a distended abdomen. There is ruminal atony and fluid is detected on abdominal ballottement and succussion.[44] Diarrhea occurs in the later stages as a result of the hyperosmolar intraluminal environment in the intestines. Hypovolemia develops when fluid continues to accumulate in the rumen as a result of the osmotic gradient that is produced by the accumulating acids. Other clinical signs include anorexia, depression, ataxia, and recumbency. Laminitis and polioencephalomalacia are possible complications.[44]

Clinical signs of SARA are nonspecific and include fluctuating dry matter intake, low body condition scores in dairy cattle, diarrhea, unexplained high cull rates due to vague health problems in dairy cows, low milk fat and lower milk production in second and higher lactation cows compared to first lactation cows.[45,46] Dry matter intake also decreases in experimentally induced SARA in dairy cattle.[47] Clinical signs of SARA in beef cattle are nonspecific and so the disease often go unrecognized.[19]

Diagnosis

Diagnosis of acute ruminal acidosis is based on the history of intake of highly digestible carbohydrates, clinical signs, and is confirmed by rumen pH of less than 5.[3] The differential diagnoses of acute ruminal acidosis include simple indigestion, parturient hypocalcemia, coliform mastitis, diffuse peritonitis and pyloric outflow failure.[3]

In dairy cattle, SARA should be diagnosed and prevented at the herd level rather than on individual animals.[45] Strategies for monitoring SARA have been developed for lactating dairy cows.[45] Testing is recommended only when a diagnosis of SARA is highly suspected. When SARA is strongly suspected in the herd a representative sample should be tested by measuring ruminal fluid pH via rumenocentesis. For most dairy herds, a sample of 12 cows is usually appropriate. The critical value for the proportion of cows with SARA should be less than 3 out of 12 (25%).[45] If the proportion is close to the critical value, retesting is recommended. The strategy of sampling 12 animals in herd with a prevalence of 5% gives a confidence of 75%.[45]

The insidious nature of SARA makes its diagnosis very difficult in feedlot cattle.[21] Monitoring intake and feeding behaviors is a necessity to detect irregularities in intake patterns because decreased feed intake may be the only sign of the problem.[19] The practice of averaging intake per pen may mask the daily intakes of individual animals, particularly as the number of animals increase per pen.[21,48]

SARA and BRD

Inflammation and immunosuppression

The incidence of bovine respiratory disease (BRD) continues to be high in feedlot cattle despite the use of vaccines and other management and husbandry practices.[49] In 1999, most feedlots (97.4%) within 12 states reported an overall BRD incidence of 14.4%, nearly five times the percentage of the next most commonly reported disease, acute interstitial pneumonia.[49] The efficacy of vaccines in

decreasing BRD morbidity and mortality is variable.[50,51,52] The variability is likely due to multiple factors such as prior vaccination, stress of commingling, transportation, environment, timing and inconsistent diets.[53] High grain diets that are provided to backgrounding and feedlot cattle likely play a significant role in increasing BRD morbidity and mortality.[5] Cattle that were fed soluble carbohydrate receiving diets had higher morbidity for bovine respiratory disease (BRD) compared to cattle fed forage based diets.[5,6] It was speculated that high grain diets were probably causing some immunosuppression that resulted in animals being more susceptible to bovine respiratory disease pathogens, but the mechanisms of immunosuppression were unknown.

D-lactic acidemia has been associated with impaired immune function.[54] Platelet activating factor(PAF)-induced reactive-oxygen-species (ROS) production and L-selectin shedding were decreased in bovine neutrophils following induction of acute ruminal acidosis.[54] Such mechanisms of immunosuppression are unlikely in cases of SARA because lactic acidemia is not a typical sequela of SARA.[22]

Subacute ruminal acidosis has been associated with increased translocation of endotoxin and biogenic amines from the alimentary tract into systemic circulation.[27,28] Endotoxin is considered to be responsible for the observed increase in acute phase protein. Increased amounts of acute phase proteins in systemic circulation are indicative of active inflammation.[27] Repeated stimulation of the innate immune system by the circulation endotoxin is thought to cause immunosuppression.[38] The rationale for this assumption comes from studies in mice where macrophages failed to produce tumor necrosis factor following secondary administration of endotoxin.[55] Such a response has not been reported in cattle.

Circulating biogenic amines in excess of 1.4 g/day cause oxidative stress in selected tissues such as the rumen, liver and pancreas in humans and livestocks.[28,56] The effect of biogenic amines on pulmonary tissues is not known. In a recent study in mice, it was demonstrated that dietary

fermentable fiber content changed the composition of the gut and lung microbiota, in particular by altering the ratio of Firmicutes to Bacteroidetes.[57] Mice fed a high-fiber diet had increased circulating levels of short-chain fatty acids (SCFAs) and were protected against allergic inflammation in the lung, whereas a low-fiber diet decreased levels of SCFAs and increased allergic airway disease.[57] Treatment of mice with the SCFA propionate led to alterations in bone marrow hematopoiesis that were characterized by enhanced generation of macrophage and dendritic cell (DC) precursors and subsequent seeding of the lungs by DCs with high phagocytic capacity but an impaired ability to promote T helper type 2 cell effector function.[57] Impaired function of T helper type 2 cells results in termination of allergic airway inflammation. The effect of SCFA or volatile fatty acids on the pulmonary tissues in cattle is not known. However, if propionate has similar effects on the bone marrow of cattle, it is possible that the enhanced generation of macrophage and dendritic cell precursors enhances inflammation of the lungs when exposed to pathogens. The effect of high grain diets and SARA on the immune system as well as on the pulmonary system in cattle needs further investigation.

Diagnosis of BRD is challenging because of lack of a gold standard and reliable markers.[58] In backgrounding/receiving and feedlot cattle, diagnosis of BRD often involves a subjective clinical assessment and measuring body temperature.[58] In general, animals that appear sick but are not exhibiting clinical signs ascribable to any other body system are considered to have BRD.[5,58] Animals experiencing SARA can potentially be misdiagnosed with BRD because both conditions often have non-specific clinical signs. The absence of ideal diagnostic markers for both conditions explain, in part, lack of concordance between treatment of clinical BRD and occurrence of lung lesions at slaughter.[60]

Economic losses associated with SARA

Economic costs associated with SARA in dairy cattle have been estimated to be US $500 million to US $1 billion annually in United States.[61] These estimates were provided in the late 1990s. The losses are associated with reduced milk production, decreased efficiency of milk production, premature culling and increased deaths. In beef calves, losses of US $10-13 per animal were attributed to reduced growth alone while condemned livers in feedlot cattle accounted for US $3 per animal.[62] The livers are condemned because of abscesses. In addition, SARA is associated with conditions that cause both direct and indirect losses, including death, reduced feed efficiency, laminitis, ruminal tympany, abomasal displacement, abomasal ulcers, and reproductive losses. The economic effects of SARA in dairy cows remain an active area of research in dairy cattle. In contrast, not much is known about its effects in beef cattle. Further studies are required to provide information about direct and indirect costs including the effectiveness of the current prevention strategies.

Effects of SARA on animal welfare and Public Health

Animal welfare concerns

One of the major animal welfare concerns in SARA affected cattle is laminitis. The association between SARA and subclinical laminitis has been described in previous studies.[63,64] The pathophysiology of laminitis is complex and not fully understood in cattle. It is hypothesized that SARA causes release of endotoxin and bioactive messengers that affect the dermis of the claws.[63,64] Endotoxin and bioactive messengers are thought to be vasoactive molecules that can alter the vasculature of the dermis of the claws.[64] It is also postulated that the endotoxin and bioactive messengers trigger production of inflammatory cytokines, which in turn activate matrix metalloproteinases (MMPs).[64] Matrix metalloproteinases disrupt connective tissues, including the suspensory apparatus of the claws.[64] Although subclinical laminitis may not show overt signs, it causes lesions that result in discomfort and pain to the animal. Examples of lesions that are caused by

subclinical laminitis include white line disease, hemorrhages of the sole, sole ulcers, and disintegration of the heel bulbs.[64]

Public health concerns

Biological contamination

Enterohemorrhagic *E. Coli* (EHEC) is a Shiga-toxin producing strain of *E. coli* that causes foodborne illness in humans.[65] The illness can range from uncomplicated diarrhea to hemorrhagic colitis and hemolytic-uremic syndrome.[65] The gastrointestinal tract of cattle is the principal reservoir of EHEC.[66] Ethanolamine, one of the biogenic amines produced in SARA, is utilized by EHEC, outgrowing commensal bacteria.[67] The use of ethanolamine also confers a marked growth advantage on *Salmonella enterica* serovar *Typhimurium* (*S. Typhimurium*) in the lumen of the inflamed intestine.[68] The implication from these two studies is that SARA might be a risk factor for contamination of food with EHEC and *S. Typhimurium.*

Physical and chemical contamination

Diarrhea is one of the clinical signs of SARA.[45] Physical contamination of hides with feces is a risk factor for physical and biological contamination of meat.[69] Diagnosis of BRD is challenging because of lack of a gold standard and reliable markers.[58] It is therefore, reasonable that some animals are incorrectly diagnosed with BRD and hence treated with antimicrobial medications for respiratory disease. There are concerns about increasing antimicrobial resistance associated with the use of antimicrobials in animals and humans.[70,71] Restrictions on antimicrobial drug use in specific institutions or premises have produced mixed results.[72,73,74] It therefore can be argued that, limiting antimicrobials' use to animals that truly need appropriate treatments has the potential to address some of these public health concerns.

Prevention of SARA

SARA is so closely linked to feeding conditions that correction of feed rations and/or feed management is essential to solve the problem.[61] The critical time for occurrence of acidosis is during the period of step-up to high-grain diets, when cattle enter feedlots.[21] Traditionally, increased dietary concentrate is fed in incremental amounts of grain over a 3- to 4-week period to minimize the risk of acidosis but the problem can still occur.[21] The type and amount of grain, type of grain processing, type and level of roughage, feed additives, pen cleaning, water availability, and prudent bunk management are important factors influencing SARA. Grains and processed grains vary in their digestibility; the higher the digestibility, the greater the chance of causing acidosis.[21] SARA can thus be prevented by blending grains that have higher rates of fermentation with those with lower digestibility of starch.[75]

Ionophores such as monensin and lasalosid are used to increase feed efficiency in the US feedlot cattle by reducing hydrogen and formic acid producers which in turn results in increased propionate production.[76] Ionophores also reduce lactate producing bacteria.[77] Monensin was successfully used to increase ruminal pH in beef cattle receiving high grain diets and in transition dairy cows.[78,79] However, in one study, monensin was not efficacious in raising ruminal pH in SARA induced dairy cows.[74] The inconsistency of monensin as a substance that increases ruminal pH could be due to differences in the concentrations of ruminal lactic acid in the studies.[74] Ruminal lactic acid concentration was reported to be above 5mM in most studies that reported the efficacy of monensin in increasing ruminal pH whereas a concentration of less than 1mM was reported in studies that reported monensin not being efficacious.[80,81] These studies suggest that monensin likely is efficacious against acute ruminal acidosis but its efficacy for the prevention of SARA might be equivocal.

Adding feed-grade alkalinizers such as magnesium oxide, sodium bicarbonate, calcium carbonate, and potassium carbonate, to the ration has been demonstrated to increase the ruminal pH and thus decrease SARA.[77] Sodium bicarbonate at 110-225g/day has been found to be the most effective.[77]

Direct fed microbes (DFM) have also been associated with an increase in ruminal pH.[82] A negative to feeding DFM is that they have been associated with decreased milk fat content. *Enterococcus faecium, Lactobacillus plantarum* and *Saccharomyces cerevisiae* are some of the microbes that have been evaluated. *Megasphaera elsdenii* (NCIMB 41125) has demonstrated the potential to be used as a direct fed microbe. In a study on the effect of drenching *Megasphaera esldenii* on health and performance of feedlot steers, average daily gain was improved in the immediate post adaptation phase.[83] In another study in high risk cattle from Oklahoma and Texas, average daily gain did not differ among calves receiving DFM vs. no DFM; however, calves receiving DFM during their first antimicrobial treatment for clinical BRD were less likely to be treated a second time within 96 h.[84] In addition, the number of calves treated twice for clinical BRD tended to be lower for calves administered DFM compared with calves not receiving DFM. These findings suggest that DFM have a potential to decrease morbidity for clinical BRD in receiving cattle.

Virginiamycin, although not an ionophore, has an antimicrobial spectrum similar to that of monensin in that Gram-positive bacteria are susceptible and Gram-negative bacteria are generally resistant.[85] Steers that received virginiamycin had higher ruminal pH and lower lactate compared to controls following a carbohydrate change, suggesting that virginiamycin might be able to control growth of lactate- producing bacteria.[86] Five antibiotics; bacitracin, methylene disalicylate, chlortetracycline, oxytetracycline, tylosin, and virginiamycin have approval for the prevention of liver abscesses in feedlot cattle.[87] Tylosin is the one that is used most widely, in combination with monensin in United States feedlots.[88] Several studies have confirmed that reduction in abscess incidence from tylosin feeding is 40% to 70%.[89,90,91]

Conclusion

The utilization of molecular techniques during the past few years has significantly improved our understanding of the pathophysiology of ruminal acidosis. The association between subacute ruminal acidosis and inflammation has been demonstrated in both beef and dairy cattle but the exact mechanisms require further evaluation. While the effects of subacute ruminal acidosis have been well studied in dairy cows, there is lack of information about its effects in beef cattle particularly its potential association with bovine respiratory disease. Deciphering the association between SARA and BRD has the potential to not only reduce the incidence of both diseases, but also improve the diagnosis and treatment of BRD in backgrounding and feedlot cattle.

References

1. Hofmann RR. Evolutionary steps of ecophysiological adaptation and diversification of ruminants: a comparative view of their digestive system. *Ecologia* 1989; 78: 443-457.

2. Nutrient Requirements of Dairy Cattle, ed 6. Washington DC, National Academy Press, 1989.

3. Radostits OM, Gay CC, Hinchcliff KW, Constable PD: Acute carbohydrate engorgement of ruminants (Ruminal lactic acidosis, rumen overload), in Veterinary Medicine (10th Ed): *A textbook of the diseases of cattle, horses, sheep, pigs and goats*. Edinburgh, Elsevier 2007, pp 314-325.

4. Plaizier JC, Krause DO, Gozho GN, McBride BW. Subacute ruminal acidosis in dairy cows: The physiological causes, incidence and consequences. *The Vet J* 2009; 176: 21-31.

5. Lofgreen GP. Nutrition and management of stressed beef calves. *Vet Clin North Am Food Anim Pract* 1983; 5:87-101.

6. Mwansa PB, Makarechian M, Berg RT. The effect of level of concentrate in feedlot diets on the health status of beef calves. *Can Vet J* 1992; 33: 665-668.

7. Mennecke B, Townsend A, Hayes, DJ, et al. A study of the factors that influence consumer attitudes toward beef products using the conjoint market analysis tool. *CARD Working Paper* 2006; 06-WP-425: 44

8. Owens FN, Goetsch AL. Ruminal fermentation, in Church (ed): *The ruminant animal: Digestive physiology and nutrition*. Englewood Cliffs, Prentice Hall, 1988, pp145-171

9. Hungate RE. Symposium on 'Nutritional implications of microbial action in the non-ruminal alimentary tract'. Microbes of nutritional importance in the alimentary tract**.** *Proc Nutri Soc* 1984; 43: 1-11.

10. Yokoyama MT, Johnson KA. Microbiology of the rumen and intestine, in Church (ed): *The ruminant animal: Digestive physiology and nutrition*. Englewood Cliffs, Prentice Hall 1988 pp 125-144.

11. Slyter LL: Influence of acidosis on rumen function, *J Anim Sci* 1976; 43:910.

12. Underwood WJ. Rumen Lactic Acidosis. Part 1. Epidemiology and Pathophysiology. *The Compendium* 1992; 14: 1127-1133.

13. Nordlund KV Garrett EF, Oetzel GR. Herd-based rumenocentesis: a clinical approach to the diagnosis of subacute rumen acidosis. *Food Anim Med & Manag* 1995; 17(8): s48-s56**.**

14. Nocek JE. Bovine acidosis: Implications on laminitis. *J Dairy Sci* 1997;80: 1005-1028.
15. Dirksen G. The rumen acidosis complex: Recent knowledge and experiences: A Review. *Tierarztl Prax* 1985; 13 (4): 501-512.

16. Krause KM, Oetzel GR. Understanding and preventing subacute ruminal acidosis in dairy herds: A review. *Anim Feed Sci Technol* 2006; 126: 215-236.

17. Gozho GN, Plaizier JC, Krause DO, Kennedy AD, Wittenberg KN. Subacaute ruminal acidosis induces ruminal lipopolysaccharide release and triggers an inflammatory response. *J Dairy Sci* 2005; 88:1399-1403.

18. Owens FN, Secrist DS, Hill WJ, Gill GR. Acidosis in cattle: a review *J Anim Sci* 1998; 76; 275-286.

19. Elam CJ. Acidosis in feedlot cattle: Practical observations. *J Anim Sci* 1976; 43: 898-901.

20. Dunlop RH, Hammand PB. D- lactic acidosis of ruminants. *Ann NY Acad Sci* 1965; 11: 1109-1130.

21. Nagaraja TG,Titgemeyer EC. Ruminal acidosis in beef cattle: The current microbial and nutritional outlook. *J Dairy SCi* (E Suupl) 2007; 90:E17-E38.

22. Goad DW, Goad CL, Nagaraja TG. Ruminal microbial and fermentative changes associated with experimentally induced subacute acidosis in steers. *J Anim Sci* 1998; 76: 234-241.

23. Tajima K, Aminov RI, Nagamine T, Matsui H, Nakamura M, Benno Y. Diet-dependent shifts in the bacterial population of the rumen revealed with real-time PCR. *App Environ Microb* 2001; 67: 2766-2774.

24. Fernando SC, Purvis 11 HT, Najar FZ, Sukharnikov LO, Krehbiel CR, Nagaraja TG, Roc BA, DeSilva U. Rumen microbial population dynamics during adaptation to a high-grain diet. *App Environ Microb*2010;76: 7482-7490.

25. Khafipour E, Li S, Plaizier JC, KrauseDO. Rumen microbiome composition determined using two nutritional models of subacute ruminal acidosis. *App Environ Microb* 2009; 75:7115-7124.

26. Dunlop RH: Pathogenesis of ruminant lactic acidosis, *Adv Vet Sci Comp Med* 16:259, 1972.

27. Khafipour E, Krause DO, Plaizier JC. A grain-based subacute ruminal acidosis challenge causes translocation of lipopolysaccharide and triggers inflammation. *J Dairy Sci 2009*; 92:1060-1070.

28. Wang DS, Zhang RY, Zhu WY, Mao YS. Effects of subacute ruminal acidosis challenges on fermentation and biogenic amines in the rumen of dairy cows. *Livestock Science* 2013;155: 262–272.

29. Garry F, McConnel C. Indigestion in ruminants, in Smith BP (4th ed): *Large Animal Internal Medicine*. St Louis, Mosby Elsevier, 2009, pp819-845.

30. Hibbard H,Peters JP, Chester ST, Robinson JA, Kotarski SF, Warren J, Croom J, Hagler Jr WM.The effect of slaframine on salivary output and subacute and acute acidosis in growing beef steers. *J Anim Sci 1995*; 73:516-524.

31. Lettat A, Nozière P, Silberberg M, Morgavi DP, Berger C, Martin C. Experimental feed induction of ruminal lactic, propionic, or butyric acidosis in sheep. *J Anim Sci*2010, 88:3041-3046**.**

32. Andersen PH: Bovine endotoxicosis—some aspects of relevance to production diseases. A review, *Acta Vet Scand Suppl* 98:141, 2003.

33. Andersen PH, Bergelin B, Christensen KA: Effect of feeding regimen on concentration of free endotoxin in ruminal fluid of cattle, *J Anim Sci* 1994; 72:487.

34. Pill AH Evidence of thiamine deficiency in calves affected with cerebrocortical necrosis, *Vet Rec* 1967; 81:178-181.

35. Forster RE, Estabrook RW. Is oxygen an essential nutrient? *Annu Rev Nutr* 1993; 13:383-403.

36. Chih CP. Roberts EL. Energy substrates for neurons during neural activity: a critical review of the astrocyte-neuron lactate shuttle hypothesis, *J Cereb Blood Flow Metab* 2003; 23:1263-1281.

37. Breves G. Hoeller H, Harmeyer J, Martens H. Thiamine balance in the gastrointestinal tract of sheep, *J Anim Sci* 1980; 51:1177-1181.

38. Zebeli Q, Metzler-Zebeli BU. Interplay between rumen digestive disorders and diet-induced inflammation in dairy cattle. *Research in Veterinary Science* 2012; 93:1099–1108.

39. Selman IE, Wiseman A, Petrie L, Pirie HM, Breeze RG. A respiratory syndrome in cattle resulting from thrombosis of the posterior vena cava. *Vet Rec*. 1974; 94(20):459-466.

40. Gudmundson J, Radostits OM, Doige CE: Pulmonary thromboembolism in cattle due to thrombosis of the posterior vena cava associated with hepatic abscessation, *Can Vet J* 1978; 19:304-309

41. Rebhun WC, Rendano VT, Dill SG, et al: Caudal vena caval thrombosis in four cattle with acute dyspnea, *J Am Vet Med Assoc* 1980; 176:1366-1369.

42. Braun U. Clinical findings and diagnosis of thrombosis of the caudal vena cava in cattle. The Veterinary Journal 2008; 175:118–125.

43. Jensen R, Mackey DR. Lactic acidosis, in Diseases of Feedlot Cattle,3rd ed Philadelphia, Lea and Febiger, 1979, pp121-131.

44. Underwood WJ. Rumen Lactic Acidosis. Part II. Clinical signs, Diagnosis, Treatment, and Prevention. *Compendium* 1992; 9(8): 1265-1270.

45. Oetzel GR, Monitoring and testing dairy herds for metabolic disease. *Vet Clin Nor Amer Food Anim Pract* 2004; 20: 651–674.

46. Colman E, Fokkink WB, Craninx M, Newbold JR, De Baets B, Fievez V. Effect of induction of subacute ruminal acidosis on milk fat profile and rumen parameters. *J Dairy Sci* 2010; 93: 4759-4773.

47. Brown MS, Krehbiel CR, Galyean ML, Remmenga MD, Peters JP, Hibbard B, Robinson J, Moseley WM. Evaluation of models of acute and subacute acidosis on dry matter intake, ruminal fermentation, blood chemistry, and endocrine profiles of beef steers. *J Anim Sci* 2000; 78:3155-3168.

48. Stock RA, R.A. Britton RA Acidosis in feedlot cattle scientific update on Rumensin/Tylan for the professional feedlot consultant, Elanco Animal Health, Indianapolis (IN) (1994), pp. A1–A16.

49. NAHMS. 2000a. Feedlot ’99 Part II: Baseline Reference of Feedlot Health and Health Management. USDA, APHIS, National Animal Health Monitoring System. Available:

http://www.aphis.usda.gov/animal_health/nahms/feedlot/downloads/feedlot99/Feedlot99_dr_PartII.pdf. Accessed April 15, 2014.

50. PerinoLJ, Hunsaker BD. A review of bovine respiratory disease field efficacy. *Bov Pract* 1997: 59-66.

51. Larson RL, Step DL. Evidence-based effectiveness of vaccination against Mannheimia haemolytica, Pasteurella multocida, and Histophilus somni in feedlot cattle for mitigating the incidence and effect of bovine respiratory disease complex. *Vet Clin North Am Food Anim Pract* 2012; 28:97-106,106e101-107, ix.

52. Morter RL, Amstutz HE. Evaluating the efficacy of a *Haemophilus somnus* bacterin in a controlled filed trial. *Bov Pract* 1983; 18:82-83.

53. Tripp HL, Step DL, Krehbiel CR, Moberly HK, Malayer JR. Evaluation of outcomes in beef cattle comparing preventive health protocols utilizing viral respiratory vaccines. *Bov Pract* 2013; 47: 54-64.

54. Concha C, Carretta DM, Alarcón P, Conejeros I, et al. Oxidative response of neutrophils to platelet-activating factor is altered during acute ruminal acidosis induced by oligofructose in heifers. J. Vet. Sci. 2014; 15(2): 217-224.
55. Zuckerman SH, Evans GF, Snyder YM, Roeder WD. Endotoxin-macrophage interaction: post-translational regulation of tumor necrosis factor expression. J. Immounology.1989; 143(4):1223-1227.

56. Fusi E, Rossi L, Rebucci R, Cheli F, et al. Administration of biogenic amines to Siaanen kids : effects on growth performance, meat quality and gut histology. *Small ruminant Res* 2004; 53: 1-7.

57. Trompette A, Gollwitzer ES, Yadava K, Sichelstiel AK, Sprenger N, Ngom-Brum C, Blanchard C, Junt T, Nocrd LP, Harris NL, Marsland BJ. Gut microbiota metabolism of dietary fiber influences allergic airway disease and hematopoiesis. *Nature Medicine* 2014: 1-8.

58. Leruste H, Brscic M, Heutinck LF, et al. The relationship between clinical signs of respiratory system disorders and lung lesions at slaughter in veal calves. *Prev Vet Med* 2012; 105: 93–100.

59. Step DL, Krehbiel CR, Depra LHA, et al. Effects of commingling beef calves from different sources and weaning protocols during a forty-two-day receiving period on performance and bovine respiratory disease. *J Anim Sci* 2008; 86: 3146–3158.

60. Wittum TE, Wollen NE, Perino LJ, Littledike TE. Relationships among treatment of respiratory tract disease, pulmonary lesions evident at slaughter, and rate of weight gain in feedlot cattle. *J Am Vet Med Assoc*. 1996; 209:814-818.

61. Enemark JMD. The monitoring, prevention and treatment of subacute ruminal acidosis. *The Vet J* 2009; 176: 32-43.

62. Stock R, Britton RL 1991.G91-1047 Acidosis. Historical materials from University of Nebraska-Lincoln Extension. http://digitalcommons.unl.edu/extensionshist/198 (accessed 06/23/2011).

63. Garrette EF, Pereira MN, Nordlund KV, Armentano LE, Goodger WJ Oetzel GR. Diagnostic Methods for the Detection of Subacute Ruminal Acidosis in Dairy Cow. *J Dairy Sci* 1999; 82: 1170–1178.

64. Greenough PR. Etiology and Pathogenesis of Metabolic Laminitis: A review of Facts and Hypotheses, in *Bovine Laminitis and Lameness: A hands-on approach*. Edinburgh, Saunders 2007, pp 44-48.

65. Law D. Virulence factors of *Escherichia coli* O157 and other Shiga toxin producing *E. coli*.J. Appl Microbiol 2000; 88: 729-745.

66. Cieslak PR, Barrett TJ, Griffin PM, Gensheimer KF, Beckett G, Buffington J, Smith MG. *Escherichia coli* O157:H7 infection from a manured garden. Lancet 1993; 342**:** 367.

67. Bertin Y, Girardeau JP, Chaucheyras-Durand F, Lyan B, Pujos-Guillot E, Harel J, Martic C. Enterohaemorrhagic *Escherichia coli* gains a competitive advantage by using ethanolamine as a nitrogen source in the bovine intestinal content. *Environ Microbiol*. 2011; 13(2):365-77.

68. Thiennimitra T, Winter SE, Winter MG, Xavier MN. et al. Intestinal inflammation allows Salmonella to use ethanolamine to compete with the microbiota. PNAS 2001; 108(42): 17480–17485.

69. Hadley PJ, Holder JS, Hinton MH. Effects of fleece soiling and skinning method on the microbiology of sheep carcases. *Veterinary Record* 1997; 140: 570-574.

70. Morley PS, Apley MD, Besser TE, Burney DP, Fedorka-Cray PJ, Papich MG, Traub-Dargatz JL, and Weese JS. Antimicrobial Drug Use in Veterinary Medicine. *J Vet Intern Med* 2005; 19: 617–629.

71. Hidron AI, Edwards JR, Patel J, et al. Antimicrobial-resistant pathogens associated with healthcare-associated infections: annual summary of data reported to the National Healthcare Safety Network at the Centers for Disease Control and Prevention, 2006-2007. *Infect Control Hosp Epidemiol* 2008; 29: 996-1011.

72. Owens RC, Fraser GL, Stogsdill P. Antimicrobial stewardship programs as a means to optimize antimicrobial use. *Pharmacotherapy* 2004; 24: 896–908.

73. Cook PP, Catrou PG, Christie JD, et al. Reduction in broadspectrum antimicrobial use associated with no improvement in hospital antibiogram. *J Antimicrob Chemother* 2004; 53: 853–859.

74. Langlois BE, Dawson KA, Leak I, Aaron DK. Antimicrobial resistance of fecal coliforms from pigs in a herd not exposed to antimicrobial agents for 126 months. *Vet Microbiol* 1988; 18: 147–153.

75. Stock RA, D.R. Brink DR, Brandt RT, et al. Feeding combinations of high moisture corn and dry corn to finishing cattle. *J Anim Sci* 1987; 65: 282–289.

76. Russell JB, Hino T. Regulation of lactate production in *Streptococcus bovis*: A spiraling effect that contributes to rumen acidosis 1985. *J Dairy Sci* 68:1712–1721.

77. Hutjens MF, Feed Additives. *Vet Clinics of Nor Amer. Food Anim Pract* 1991; 7(2): 525–540.

78. Nagaraja, T. G., T. B. Avery, S. J. Galitzert, and D. L. Holman. Effect of ionophore antibiotics on experimentally induced lactic acidosis in cattle. *Am J Vet Res* 1985; 46:2444–2452.

79. Green BL, McBride BW, Sandals WD, Leslie KE, Bagg R, Dick P The impact of the monensin controlled release capsule upon subclinical acidosis in the transition dairy cow. *J Dairy Sci* 1999; 82: 333–342.

80. Osborne JK, Mutsvangwa T, Alzahal O, Duffield TF, Bagg R, Dick P, Vessie G, McBride BW. Effects of Monensin on Ruminal Forage Degradability and Total Tract Diet Digestibility in Lactating Dairy Cows During Grain-Induced Subacute Ruminal Acidosis. *J Dairy Sci* 2004; 87:1840–1847.

81. Owens FN, Secrist DS, Hill WJ, Gill DR. Acidosis in cattle: A review. *J Anim Sci* 1998; 76: 275–286.

82. Nocek, J.E., Kautz, W.P. Direct-Fed Microbial Supplementation on Ruminal Digestion, Health, and Performance of Pre- and Postpartum Dairy Cattle. *J Dairy Sci* 2006; 89: 260–266

83. Leeuw KJ, Siebrits FK, Henning PH et al. Effect of *Megasphaera esdenii* NCMB 41125 drenching on health and performance of steers fed high and low roughage diets in the feedlot. *South African J Anim Sci* 2009; 39: 337-348.

84. Krehbiel, CR, Berry BA, Reeves JM, Gill DR, Smith RA, Step DL, Choat WT, Ball RL. 2001. Effects of feed additives fed to sale barn-origin calves during the receiving period: Animal performance, health and medical costs. Okla. Agr. Exp. Stn. Available: http://www.ansi.okstate.edu/research/research-reports-1/2001. Accessed March 30 2014

85. Nagaraja TG, Taylor MB. Susceptibility and resistance of ruminal bacteria to antimicrobial feed additives. *Appl. Environ. Microbiol.*1987; 53:1620-1625.

86. Coe ML, Nagaraja TG, Sun YD, et al. Effect of Virginiamycin on ruminal fermentation in cattle during adaptation to a high concentrate diet and during an induced acidosis. *J. Anim. Sci* 1999; 77:2259–2268.

87. Combined compendium of food additive specifications. Analytical methods, test procedures and laboratory solutions used by and referenced in the food additive specifications 2005; 4: 1-158.

88. Nagaraja TG, Lechtenberg,KF, Liver Abscesses in Feedlot Cattle. *Veterinary Clinics of North America: Food Animal Practice* 2007; 23(2): 351-369.

89. Brink DR, Lowry SR, Stock RA, et al. Severity of liver abscesses and efficiency of feed utilization of feedlot cattle. *J Anim Sci*, 1990; 68: 1201–1207.

90. Brown H, Bing RF, Grueter HP, et al. Tylosin and chlortetracycline for the prevention of liver abscesses, improved weight gains and feed efficiency in feedlot cattle. *J Anim Sci* 1975; 40: 207–213.

91. Tan ZL, Lechtenberg KF, Nagaraja TG, et al. Serum neutralizing antibodies against Fusobacterium necrophorum leukotoxin in cattle with experimentally induced or naturally developed hepatic abscesses. *J Anim Sci*.1994; 72: 502–508.

CHAPTER II

SUBACUTE RUMINAL ACIDOSIS IN RECENTLY WEANED BEEF CATTLE UNDERGOING PRECONDITIONING

Introduction

Subacute ruminal acidosis (SARA) is a metabolic condition in ruminants, primarily cattle, caused by consumption of highly fermentable carbohydrates and characterized by repeated episodes of ruminal pH between 5.2 and 5.6[1,2,3]. Highly digestible carbohydrates can be in the form of concentrates or forages including alfalfa. Evidence suggests that SARA can also occur in dairy cows on grass pasture.[4]

When ruminal pH decreases to 5.6 or less, changes occur in the ruminal flora and digestion. Total viable anaerobic and amylolytic bacterial counts were shown to be increased in experimentally induced SARA in steers.[5] Lactate-utilizing bacterial counts were also increased in that study.

Although limited, recent molecular studies appear to support the earlier culture based experiments that demonstrated decreases in cellulolytic bacteria such as *Ruminococcus* spp with a concomitant shift to lactate-utilizing bacteria such as *Megasphaera elsdenii* and *Selemonas ruminantium* when cattle were transitioned from forage based diets to grain diets.[5,6,7]

The decrease in pH observed in SARA is a result of increased total volatile fatty acid (VFA) concentration.[8] There is also a shift in the proportion of VFAs in the ruminal fluid of cattle with SARA. The molar proportions of butyrate and propionate increase while acetate decreases in cases of SARA.[8,9,10] Increasing proportions of butyrate and propionate by the ruminal bacteria stimulate proliferation of rumen papillae.[3] An exaggeration of this process results in parakeratosis of the rumen papillae, a situation that predisposes the mucosa to trauma and also prevents absorption of VFAs resulting in further decreases in pH and poor production.[11]

Strategies to prevent or minimize the incidence SARA in feedlot cattle are available and widely practiced. These practices include providing starter or receiving diets that contain no less than 25% fiber as dry matter intake, following proper stocking density, providing adequate bunk space, incorporating ionophores in rations, feeding total mixed rations and adding pH buffers to the feed[3,12,13,14] The effectiveness of these strategies in receiving beef cattle is unclear because studies that evaluate occurrence of SARA in beef cattle undergoing preconditioning have not been published or reported.

It has been observed that the occurrence of bovine respiratory disease (BRD) increases when cattle are introduced to high grain diets.[15] Cattle that were fed diets containing 75% concentrates and 25% forage per dry matter basis had higher morbidity for BRD compared to cattle that were fed hay.[15,16] The case definition of BRD diagnosis was unclear in both studies but all animals that appeared sick were considered as having BRD. Given such a criterion, it is reasonable to suspect that some animals were misdiagnosed. The exact cause of increase in BRD morbidity in cattle fed

high grain diets is unknown; it is speculated[16] that high grain diets are associated with immunosuppression, resulting in cattle becoming more susceptible to BRD.

The purpose of this study was to evaluate whether SARA occurs in cattle that are receiving preconditioning diets and determine the practicality of ruminal fluid analysis via sample collection by rumenocentesis as a diagnostic tool under typical management conditions for backgrounding or feedlot operations.

Materials and methods

The study was approved the Oklahoma State University, Institutional Animal Care and Use Committee (IACUC). Sixty (60) out of 210 recently weaned, commingled beef cattle, average weight 221.35 kg (488lb) were randomly enrolled in the study. The animals were on native pastures prior to purchase and enrolment into the study. All animals were processed and managed according to the protocols at the Willard Sparks Beef Research Center, Oklahoma State University. Processing included dehorning (8 animals), castration (7 animals), deworming using injectable ivermectin[i] subcutaneously (all animals), vaccination using a modified live viral vaccine[ii] subcutaneously, and antimicrobial metaphylaxis using tilmicosin[iii] subcutaneously (all animals) for the prevention of BRD in these high-risk cattle. Cattle were fed a diet that met or exceeded the NRC guidelines. The diet contained cracked corn, corn gluten, and sorgum Sudan hay. In addition, minerals, vitamins, tylosin[iv] and monensin[v] were added to the feed. Overall, the feed was 25% fiber and 75% concentrate on a dry matter basis. The feed was provided twice a day as a total mixed ration. Fresh water was provided *ad libitum*.

Footnotes i-Ivermectin Plus®, ii-Pyramid-5®, iii-Micotil®, iv-Tylan®, v-Rumensin®

Rumenocentesis and ruminal fluid analysis

Rumenocentesis was performed according to a previously described procedure in dairy cows with minor modifications.[17] The procedure was performed weekly for four weeks. The first samples (baseline) were collected on the day of processing. The animal was restrained in a squeeze chute. The person performing the rumenocentesis identified a 10cm X 10cm area located approximately 15 cm caudoventral to the costochondral junction of the last rib on a line parallel with the proximal aspect of the stifle. The area was clipped, scrubbed with a povidone-iodine scrub, and sprayed with 70% isopropyl alcohol. Tail jacking was performed and a 16 gauge, 4 inch stainless steel needle was inserted into the ventral sac of the rumen and a 10 mL syringe was used to aspirate a minimum of 2 mL of ruminal fluid. Ruminal pH was measured immediately using a hand-held digital pH meter (Horiba Instruments Incorporated, Irvine, CA). Approximately 1mL of the fluid was stored for laboratory use. The remaining fluid was used for sedimentation, methylene blue reduction test and for microscopic examination. For sedimentation, approximately 0.5 ml of ruminal fluid was transferred into a glass tube and observed for the time it took for the fluid to sediment. For the methylene blue reduction test, 0.1mL of methylene blue was added to a vial containing approximately 0.5mL of ruminal fluid and the time it took for the color to change from blue to green was noted. For protozoal viability, a drop of ruminal fluid was put on a warm (37°C) microscope slide and covered with a cover slip. The slides were evaluated using a light microscope with a 10X objective lens. The amount of ruminal fluid was too little to objectively quantify the protozoa; a combination of semi-quantitative and qualitative approach was used instead. Three categories were created to grade the protozoa viability. Grade 1 was assigned to protozoa that were few in numbers and had sluggish motility, grade 2 was for moderate numbers and fair to good motility, grade 3 was for large numbers and excellent motility.

Clinical observation for BRD diagnosis

An experienced clinician observed the animals daily to identify clinical signs attributable to BRD or other diseases using the DART ™ system with some modifications[18]. Specifically, the subjective criteria used to indicate clinical BRD included depression (D), abnormal appetite (A), and respiratory signs (R). Signs of depression included depressed attitude, hanging head, glazed or sunken eyes, slow movement, arched back, difficulty getting up from lying down, knuckling or dragging toes when walking, and stumbling when moving. Signs of abnormal appetite included completely off feed, eating less than expected, slow eating, lack of fill (sunken left paralumbar fossa), and obvious body weight loss. Respiratory signs included obvious dyspnea (labored breathing), extended head and neck (orthopnea), and audible noise when breathing. The evaluator also assigned a severity score of 1 to 4, where 1 was assigned for mild, 2 for moderate, 3 for severe, and 4 for moribund (steer would not rise from recumbency; assistance was needed) during their evaluation. The fourth criterion used to determine if antimicrobial therapy was needed on an individual animal basis was an objective measurement of rectal temperature with a digital thermometer (GLA M750, Metermall, USA). Any animal with a rectal temperature of 40°C or greater received an antimicrobial according to label directions. In addition, any animal with a severity score of 3 or 4 was treated with an antimicrobial regardless of the rectal temperature.

DNA Isolation and quantitative real time PCR

DNA was isolated from ruminal fluid samples using a DNA isolation kit (QIAamp Stool Mini Kit, Qiagen) and following the manufacturer's protocol. Briefly, lysis buffer was added to the 200uL samples that had been frozen. The mixture was incubated at 70° C for 5 minutes. Samples were centrifuged and an InhibitEX tablet was added to each sample to bind inhibitors. Proteinase K, buffer and ethanol were added and samples were incubated at 70° C for 10 minutes. Samples were loaded into spin columns and washed twice with separate solutions before DNA was eluted with 100 uL of elution buffer. The isolated DNA was stored at -20° C until further analysis.

Quantitative real time PCR was performed to quantify specific bacteria based on their unique 16S DNA sequences. Quantitative analysis of population changes of selected bacterial species were conducted using real-time polymerase chain reaction (qRT-PCR). Ten animals were randomly selected from the animals that had at least one recorded ruminal acidosis episode, pH≤ 5.6 and another ten were randomly selected from the animals that had never had a recorded acidosis episode. The selected bacteria included *Prevotella ruminocola*, *Ruminococcus flavifaciens*, *Fibrobacter succinogenes*, *Selenomonas ruminantium*, *Megasphaera elsdenii*, *Streptococcus bovis*, and *Lactobacillus acidophilus*.

Each qPCR assay was performed using a SYBR green assay kit (Roche Diagnostics, Indianapolis, IN). The dynamic range and PCR efficiency of each assay were evaluated using positive controls. A 15µl reaction mixture contained 5 uM (each) forward and reverse primer, 7.5µl Sybr Green Master mixture (Roche Diagnostics), and 30 ng of ruminal fluid DNA. Thermal cycling conditions were 95°C for 10 min, followed by 50 additional cycles of 95°C for 30 s, 54°C for 30 s, and 72°C for 40 s or 1 min, and finally, a melting curve was prepared. A universally conserved fragment of 16S was used for normalization. The qPCRs were performed using a MyIQ RT-PCR instrument (BioRad). Agarose gel electrophoresis was used to verify amplification of the correct products. The primers and PCR conditions are shown in Table 1. Relative quantification of bacterial population changes was performed using the comparative threshold cycle (*CT*) method, as described previously.[5]

Statistical analyses

The SPSS® analytical software was used for statistical analyses. The differences in ruminal pH among the four sampling days were analyzed by ANOVA multivariate analysis. The relationships between ruminal pH and sedimentation time or protozoa viability were analyzed by Spearman's rho. Initial analysis of fold changes revealed that there was tremendous variation of bacterial concentrations among the cattle and the data did not follow a normal distribution. Data was

therefore analyzed by a Wilcoxon signed-rank test. Results were considered statistically significant if the p-value was less than 0.05. A decision was made to put any animals that had at least one acidotic group in the acidosis group for comparison with the non-acidotic group. The non-acidotic group was comprised of animals that never had a recorded acidosis (pH≤5.6) episode. This group had a mean ruminal pH of 6.2.

Results

Rumenocentesis

All sixty (60) animals were sampled at arrival and weekly for 4 weeks for a total of five samples per animal. The average time for the whole sampling procedure was approximately 3 minutes per animal. Three steers developed very subcutaneous swellings (0.5-1.5 cm). The 3 animals that developed the swelling were bright alert and responsive throughout the study period. All the swellings resolved without any intervention. None of the 3 animals was ever identified as showing clinical signs of disease.

Ruminal pH

An animal was considered to have ruminal acidosis if the ruminal fluid pH was ≤ 5.6.[3] Ruminal pH varied with days of sampling (p=0.0001). The number of animals that were acidotic (pH<5.6) increased for three weeks and then decreased on the fourth week. One animal was acidotic on initial sampling, six steers were acidotic on day 7, 12 (20%) steers were acidotic on day 14, 13 (22%) steers were acidotic on day 21, and one animal was acidotic on day 28. The pH data is presented in Fig. 1

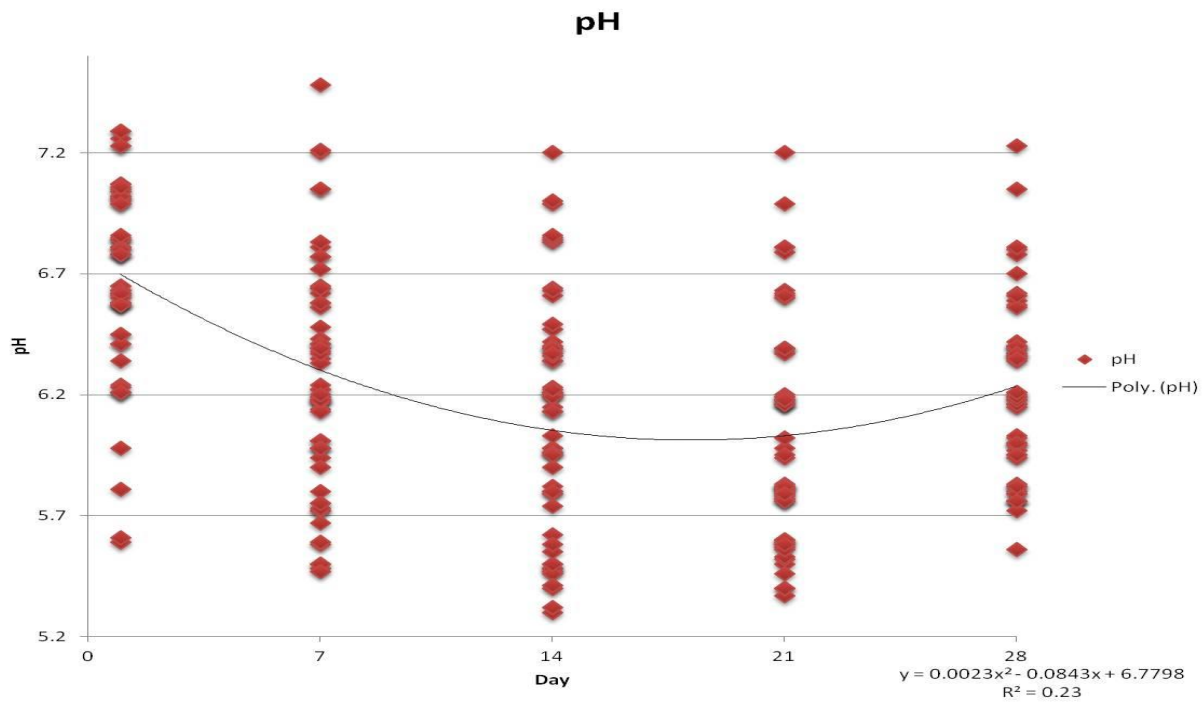

Fig.1. Ruminal fluid pH of animals that were sampled on arrival and once weekly for four weeks pH values of all the animals on days 0,7,14,21, and 28. The line describes the change in average ruminal fluid pH from day 0 to day 21.

Sedimentation time

The mean sedimentation time was 62 seconds, range 40 seconds to 4 minutes 21 seconds. The mean sedimentation time was low for all samples regardless of the pH. There was a significant positive correlation between pH and sedimentation time; as pH increased, sedimentation time also increased. The correlation coefficient for this was 0.467 ($p<0.01$).

Protozoal viability

Overall, protozoa were viable in all samples throughout the study period. Three different sizes (small, intermediate, and large) of protozoa were observed. The pH was negatively correlated with protozoal viability, with a coefficient of -0.550 ($p<0.01$). The acidotic group had more viable protozoa compared to the non-acidotic group as shown in Fig. 2.

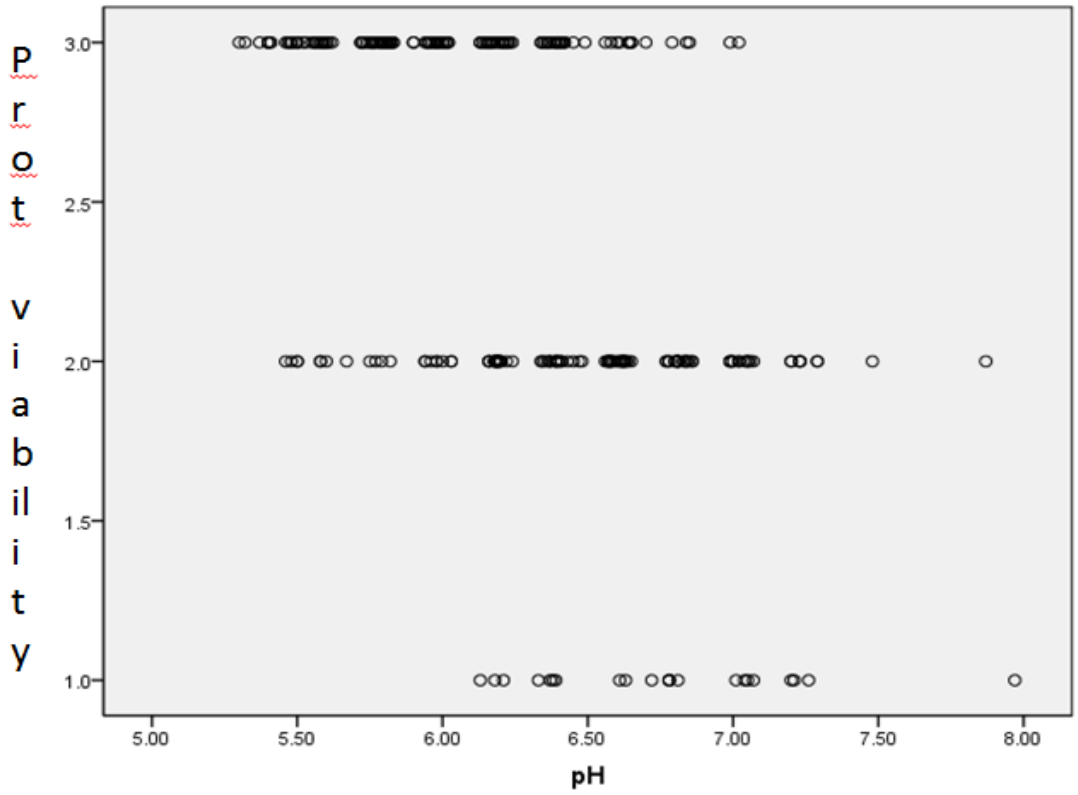

Fig. 2: Viability of ruminal protozoa from animal with varying ruminal pH levels
pH values of the ruminal fluid that was analyzed and categorized into grades 1, 2 and 3.

Primer	Sequence 5'-3'	Product size (bp)	Annealing Temp (°C)
Prevotella ruminocola-For *Prevotella ruminocola*-Rev	GGTTATCTTGAGTGAGTT CTGATGGCAACTAAAGAA	485	54
Selomonas ruminantium-For *Selomonas ruminantium*-Rev	TGCTAATACCGAATGTTG TCCTGCACTCAAGAAAGA	513	54
Megasphaera elsdenii-For *Megasphaera esldenii*-Rev	GACCGAAACTGCGATGCTAGA TCCAGAAAGCCGCTTTCGCCAC T	128	54
Streptococcus bovis-For *Streptococcus bovis*-Rev	ATTCTTAGAGATAGGGTTTCTC TT ACCTTATGATGGCAACTAACAA TA	134	54
Lactobacillus acidophilus-For *Lactobacillus acidophilus*-Rev	GTTCCTTCGGGGACACTAAGAC AG TCCCGAGTTAGGCCACCGGCTT TG	450	54
Fibrobacter succinogenes-For *Fibrobacter succinogenes*-Rev	GGTATGGGATGAGCTTGC GCCTGCCCCTGAACTATC	445	54
Ruminococcus flavefaciens-For *Ruminococcus flavefaciens*-Rev	GGACGATAATGACGGTACTT GCAATCYGAACTGGGACAAT	295	54
16S-For 16S-Rev	AGACTTTGATCCTGGCTCAG TGCTGCCTCCCGTAGGAGT	805	54

Table1. Primer sequences used for quantitative real time PCR. The fold change in expression of specific 16S genes was compared between the acidotic group and the non-acidotic group. Ten animals were in each group.

Real time PCR

There were no statistically significant differences in bacterial concentrations, as represented by the ranks of fold changes between the acidotic and non-acidotic group (p-values are shown in Table 2). The relative concentrations of amilolytics (*Streptococcus bovis* and *Lactobacillus acidophilus*) tended to increase over time in the acidotic group, and either tended to decrease or modestly increase in the non-acidotic group. The relative proportion of Fibrolytics (*Fibrobacter succinogenes, Prevotella ruminocola* and *Ruminococcus flavefaciens*) decreased or modestly increased during the first week and stabilized over time in the acidotic group. In contrast, the relative proportion of fibrolytics tended to decrease or modestly increase over time in the non-acidotic group except *Prevotella ruminocola*. Lactilytics (*Megasphaera elsdenii* and *Selomonas ruminantium*) showed a relative increase during the first week and remained so over time in the acidotic group but, decreased or modestly increased during the entire study period in the non-acididotic group.

Bacteria	Day 7 Acidotic group fold change vs. Non-acidotic group fold change P- value	Day 14 Acidotic group fold change vs. Non-acidotic group fold change P- value	Day 21 Acidotic group fold change vs. Non-acidotic group fold change P- value
Lactobacillus acidophilus	0.79	0.67	0.31
Streptococcus bovis	0.67	0.24	0.43
Megasphaera elsdenii	0.064	0.089	0.242
Selomonas ruminantium	0.47	0.97	0.67
Prevotella ruminocola	0.73	0.1	0.27
Ruminococcus flavefaciens	0.79	0.79	0.21
Fibrobacter succinogenes	0.14	0.88	0.6

Table 2. qRT-PCR results showing P- values of the differences in 16S expression of selected bacteria between the acidotic group and the non-acidotic group.

Bacterial populations and ruminal pH relationship

The relationships between changes in ruminal pH and changes in the relative proportions of the measured bacteria are shown in Table 3. The weekly change in *Lactobacillus acidophilus* 16S expression was not significantly associated with weekly pH changes (P= 0. 734). The change in *Lactobacillus acidophilus* 16S expression of over the entire study period was not significantly associated with pH changes (P= 0.724). The weekly change in *Streptococcus bovis* 16S expression was not significantly associated with weekly pH changes (P= 0. 281). The change in *Streptococcus bovis* 16S expression of over the entire study period was not significantly associated with pH changes (P= 0.41). The weekly change in *Ruminococcus flavefaciens* 16S expression was not significantly associated with weekly pH changes (P= 0. 89). The change in *Ruminococcus flavefaciens* 16S expression of over the entire study period was not significantly associated with pH changes (P= 0.833). The weekly change in *Selomonas ruminantium* 16S expression was not significantly associated with weekly pH changes (P= 0. 604). The change in *Selomonas ruminantium* 16S expression of over the entire study period was not significantly associated with pH changes (P= 0.346).

Interestingly, the weekly change in expression of *Fibrobacter succinogenes* 16S was positively correlated with the weekly changes in pH (p= 0.011). Likewise, the change in *Fibrobacter succinogenes* 16S expression over the entire study period was not significantly associated with pH changes (P=0.029). The weekly changes in pH were positively correlated with weekly changes in *Prevotella ruminocola* 16S expression (P=0.035). However, the change in *Prevotella ruminocola* 16S expression over the entire study period was not significantly associated with overall pH changes (p=0.061). Similarly, the weekly changes in pH were positively correlated with changes in *Megasphaera elsdenii* 16S expression (P=0.011) but, the change in *Megasphaera elsdenii* 16S expression was not significantly associated with overall pH changes (p=0.263).

Bacteria	Weekly pH change correlation with fold change rank P- value	Overall pH change correlation with overall fold change rank P- value
Fibrobacter succinogenes	0.011	0.029
Prevotella ruminocola	0.035	0.061
Ruminococcus flaveficiens	0.890	0.833
Megasphaera elsdenii	0.011	0.263
Selomonas ruminantium	0.604	0.346
Streptococcus bovis	0.281	0.410
Lactobacillus acidophilus	0.734	0.724

Table 3. Results of the regression analyses of changes in ruminal pH and changes in relative bacterial population changes

Health and performance

There were no clinically significant problems associated with the rumenocentesis procedure. Seventeen (28%) steers were observed and met the case definition for treatment of bovine respiratory disease (BRD) during the study. One animal died on day 11 from bronchopneumonia. Treatment for BRD was not associated with acidosis (p=0.144). None of the animals that were treated for BRD had a recorded episode of ruminal acidosis during the study. The average daily gain (ADG) for the animals that experienced at least one recorded acidosis episode (acidotic group) was 1.02 kg (2.24 lb) and those that did not have a recorded episode of acidosis during the study period (non-acidotic group) was 0.58 kg (1.46 lb), respectively. These ADG values were statistically significant, p=0.006.

Discussion

Rumenocentesis was successfully performed in all the steers under the typical field settings. The absence of clinically significant complications in this study is consistent with the data from dairy cattle.[17]

The number of animals that were acidotic increased for the first three weeks and then decreased on the fourth week. This study is consistent with previous reports that showed that cattle frequently experience acidosis when introduced to highly digestible carbohydrates[3,19] but will adopt over time.[5] This study provides more information about the estimated proportion of cattle that experience episodes of SARA during preconditioning. Twenty percent (20%) of the steers sampled were acidotic on day 14 and twenty-two percent (22%) were acidotic on day 21.We could not estimate the duration of acidosis in these animals because there was no continuous monitoring of ruminal pH. The steers were sampled 4-6 hours after feeding; it is therefore possible that some animals might have been acidotic in the earlier hours but pH increased to above 5.6 by the time of sampling. This possibility, combined with the fact that samples were

only collected once a week, suggest that this is a minimum proportion that could be expected to suffer SARA, and the true incidence may be notably higher. However, it is still important that a significant proportion of the animals experienced SARA during the first 3 weeks.

It is interesting to note that prevalence of SARA increased for the first three weeks and then decreased by the fourth week. These changes were likely reflections of the adjustments of ruminal microbiota. Cattle that were fed step-up diets for four weeks had changes in ruminal microbiota that were attributed to acidosis although ruminal pH was not measured in those animals.[5] Another notable finding from the current study was that acidotic steers on day 7 were likely to be acidotic on days 14 and 21. This finding suggests animals that experience ruminal acidosis are likely to have repeated acidotic episodes for the first three weeks of the receiving period. The current strategies in the field to prevent ruminal acidosis as outlined in the introduction may need to be further evaluated. At least in this study, the strategies are good at preventing acute ruminal lactic acidosis but not SARA.

There was no statistically significant association between being acidotic on at least one sampling point and treatment for clinical BRD. Previous studies have reported that BRD morbidity increased in cattle that were introduced to high grain diets, presumably as a result of ruminal acidosis and immunosuppression.[15,16] However, ruminal pH was not measured in these studies and so the level of acidosis in these animals was not truly known. Furthermore, the mechanisms of immunosuppression in animals experiencing SARA have not been elucidated. Experimental studies in both beef and dairy cattle have demonstrated that SARA is associated with chronic systemic inflammation.[20] It is suspected that transition diets cause constant inflammatory response that causes metabolic changes resulting in dairy cows being more susceptible to periparturient diseases.[21] In dairy cows, SARA is associated with periparturient conditions such as displaced abomasum, hypocalcemia, and decreased milk fat.[3] Information is lacking in beef cattle and demonstrating the occurrence of SARA in recently weaned beef cattle undergoing

preconditioning is one of the steps towards understanding the effect of SARA on health of beef cattle. Management of beef cattle is different from dairy cattle and so there are likely to be unique problems related to SARA in beef cattle. In the current study, ruminal fluid samples were not obtained at the time of identifying steers for BRD treatment and therefore it may not be surprising that we did not find an association between SARA and BRD treatment. A separate experiment in which ruminal pH is measured at the time of pulling morbid cattle is necessary. Thomson et al 2009, measured ruminal pH of animals that had for a clinical BRD diagnosis and BRD incidence was independent of ruminal pH.[22] However, in that study, the animals were transported from the feedlot facility to the hospital where ruminal fluid pH was measured and so the ruminal pH might have changed during the transport time. Alternatively, differences are more likely to be recorded when there is constant monitoring of ruminal pH.

The ADG for the steers that would have at least one recorded episode of acidosis (acidotic group) was higher than that of the steers that did not have a recorded episode of acidosis (non-acidotic group), although there was no statistical difference. Our inability to monitor ruminal pH constantly makes it difficult to make specific conclusions on performance of these animals

Sedimentation time provides a rough estimate of the fermentation of ruminal ingesta.[8] Normal ruminal fluid sedimentation time ranges from 4-8 minutes and ruminal fluid with acidosis will have sedimentation time of less than 4 minutes.[8] In the current study there was no difference in ruminal fluid sedimentation time between the acidotic group time and the non-acidotic group time, and both groups had average sedimentation time that was less than 4 minutes. This finding was anticipated because both groups received the same diet and sedimentation test is likely to be nonspecific for a diagnosis of SARA. A sedimentation time is likely a good diagnostic test for acute ruminal acidosis but its accuracy for SARA diagnosis appears unclear.

Protozoal viability was also assessed in this study in order to determine if it could be used for a diagnosis of SARA. There was a negative correlation between ruminal fluid pH and protozoa viability. The information on protozoa viability in SARA in the literature is inconclusive.[23] It is suggested that the viability of protozoa initially decreases due to low ruminal pH but protozoa thrive at a pH of 5.5 in the rumen of limit-fed cattle consuming high-starch purified diets.[23] In the current study, protozoa were more viable in animals experiencing acidosis than in non-acidotic animals suggesting that the level of acidosis is not low enough to cause death of protozoa. It is, however, important to point out that the control group in this study consisted of animals that were not experiencing acidosis but were getting the same diet as the acidotic group. Different results might be obtained or expected if animals that are on forage diets are used as controls.

Studies in both dairy and beef cattle have demonstrated that the profile of ruminal microbiome change when high grain diets are fed.[5] The diversity of bacteria significantly decreases when high grain diets are introduced, likely because of ruminal acidosis.[5] In general, plant cell wall degraders decrease with increasing concentrates in the diets whereas starch degraders and lactate utilizers increase. However, results from studies on quantities of ruminal bacteria from cattle fed high grain diets have not always been consistent with regards to specific bacteria. There were significant population increases in *Streptococcus bovis* (amilolytic) *Megasphaera elsdenii* (lactilytic), *Selomonas ruminantium* (lactilytic), and *Prevotella bryantii* (amilolytic) whereas *Butyrivibrio fibrosolvens* and *Fibrobacter succinogenes (both fibrolytics)* decreased in cattle that were fed step-up diets.[5] In that study, ruminal pH was not measured but it was assumed that the changes in ruminal microbiome were a result of ruminal acidosis and adaptation.[5] In the current study, there was a preponderance of repeated SARA episodes after recording of an episode of acidosis (pH$\leq$ 5.6). There were no statistically significant differences in the relative proportions of the measured bacteria in the current study. The lack of statistically significant differences may be attributed to the single diet that the all steers were receiving. Steers that receive the same diet

are likely to respond similarly, but at different degrees such that numerical or clinically significant difference might be observed but not attain statistical significance. Our inability to monitor ruminal pH constantly might also have resulted in the misclassification of some steers. Nevertheless, the changes in relative proportions of various bacterial species suggested that ruminal pH plays a role in ruminal bacteria population dynamics. This relationship is supported by the regression analyses that showed positive correlation between ruminal pH and the relative proportion of bacteria as represented by analysis of ranking of 16S fold change of *Fibrobacter succinogenes, Prevotella ruminocola and Megasphaera elsdenii* (Table 3). *Fibrobacter succinogenes* and *Prevotella ruminocola* are fibrolytics which means that their growth is mainly depended on utilization of cellulose.[23] They are both sensitive to decreases in pH.[23] The positive correlation between the relative proportion of *Fibrobacter succinogenes* and *Prevotella ruminocola* that was observed in the current study provide more evidence that SARA occurs in beef cattle that are fed typical receiving diets. The current study also provides evidence that there can be a huge variability in individual animal's response to the receiving diets. This variability suggests that there might be a need to do further studies on ruminal microbiome in receiving cattle using more study units than those that have been used in previous studies. The impact of SARA in beef cattle also needs further investigation.

Conclusion

This study clearly reveals that recently weaned beef cattle receiving preconditioning rations at a backgrounding facility experience SARA. The fact that animals in this study experienced SARA despite control measures such as bunk management, feeding total mixed ration and addition of monensin suggests that these measures may not be enough to prevent SARA and potential complications may occur. It is important to evaluate potential complications as they relate to BRD occurrence or laminitis occurrence; particularly with the increasing use of metabolic implants and beta agonists in beef cattle.

References

1. Cooper R, Klopfenstein T. 2006. Effects of Rumensin and feed intake variation on ruminal pH. Update on Rumensin / Tylan /Micotyl for the Professional Feedlot Consultant. Elanco Animal Health, Greenfield, IN.

2. Gozho GN, Plaizier JC, Krause DO, Kennedy AD, Wittenberg KM. Subacute Ruminal Acidosis Induces Ruminal Lipopolysaccharide Endotoxin Release and Triggers an Inflammatory Response. *J. Dairy Sci*. 2005; 88:1399–1403.

3. Nagaraja TG,Titgemeyer EC. Ruminal acidosis in beef cattle: The current microbial and nutritional outlook. *J. Dairy.Sci* (E Suupl)2007; 90:E17-E38

4. O'Grady L,Doherty ML, Mulligan FJ. Subacute ruminal acidosis (SARA) in grazing Irish dairy cows. *The Veterinary Journal* 2008;176 : 44–49.

5. Fernando SC, Purvis 11 HT, Najar FZ, Sukharnikov LO, Krehbiel CR, Nagaraja TG, Roc BA, DeSilva U. Rumen microbial population dynamics during adaptation to a high-grain diet. *Applied and Environmental Microbiology*. 2010;76:7482-7490

6. Tajima K, Aminov RI, Nagamine T, Matsui H, Nakamura M, Benno Y. Diet-dependent shifts in the bacterial population of the rumen revealed with real-time PCR. Applied and *Environmental Microbiology* 2001; 67: 2766-2774.

7. Khafipour E, Li S, Plaizier JC, KrauseDO. Rumen microbiome composition determined using two nutritional models of subacute ruminal acidosis. *App Environ Microb* 2009; 75:7115-7124.

8. Garry F, McConnel C. Indigestion in ruminants, in Smith BP (4th ed): *Large Animal Internal Medicine*. St Louis, Mosby Elsevier, 2009, pp819-845.

9. Hibbard H, Peters JP, Chester ST, Robinson JA, Kotarski SF, Warren J, Croom J, Hagler Jr WM.The effect of slaframine on salivary output and subacute and acute acidosis in growing beef steers. *J Anim Sci 1995*; 73:516-524.

10. Lettat A, Nozière P, Silberberg M, Morgavi DP, Berger C, Martin C. Experimental feed induction of ruminal lactic, propionic, or butyric acidosis in sheep. *J Anim Sci* 2010, 88:3041-3046**.**

11. Slyter LL: Influence of acidosis on rumen function, *J Anim Sci* 1976; 43:910

12. Nagaraja, T. G., T. B. Avery, S. J. Galitzert, and D. L. Holman. Effect of ionophore antibiotics on experimentally induced lactic acidosis in cattle . *Am J Vet Res* 1985; 46:2444–2452.

13. Russell JB, Hino T. Regulation of lactate production in *Streptococcus bovis*: A spiraling effect that contributes to rumen acidosis 1985. *J Dairy Sci* 68:1712–1721.

14. Stock RA, D.R. Brink DR, Brandt RT, et al. Feeding combinations of high moisture corn and dry corn to finishing cattle. J Anim Sci 1987; 65:282–289.

15. Lofgreen GP. Nutrition and management of stressed beef calves.*Vet.Clin.North.Am.*1983; 5:87-101

16. Mwansa PB, Makarechian M, Berg RT. The effect of level of concentrate in feedlot diets on the health status of beef calves. *Can Vet J* 1992; 33: 665-668.

17. Garret EF, Pereira MN, Nordlund KV, et al Methods for the Detection of Subacute Ruminal Acidosis in Dairy Cows. *J Dairy Sci* 1999; 82:1170–1178

18. Step DL, Krehbiel CR, Depra HA et al. The effects of commingling beef calves from different sources and weaning protocols during a forty- two -day receiving period on performance and bovine respiratory disease. *J Anim Sci* 2008, 86:3146-3158.

19. Khafipour E; Krause DO, Plaizier JC. A grain-based diet subacute ruminal acidosis challenge causes translocation of lipopolysaccharide and triggers inflammation. *J. Dairy Sci*. 2009a; 92:1060-1070.

20. Ametaj BN, Koenig KM, Dunn, Yang WZ, Zebeli Q, Beauchemin KA. Backgrounding and finishing diets are associated with inflammatory responses in feedlot steers. *J. Anim Sci* 2009; 87:1314-1320.

21. Zebeli Q, Metzler-Zebeli BU, Ametaj BN. Meta-analysis reveals threshold level of rapidly fermentable dietary concentrate that triggers systemic inflammation in cattle. *J. Dairy Sci*. 2012; 95 :2662–2672.

22. Thomson DU. Monitoring of rumen pH in cattle pulled for bovine respiratory disease complex with normal or elevated rectal temperatures in a commercial feedlot. In Proceedings of the Fortieth Annual Conference, American Association of Bovine Practitioners, Vancouver, British Columbia, Canada, 20-22 September, 2007. AABP 273.

23. Owens FN, Goetsch AL. 1988 .Ruminal Fermentaion in The ruminant animal: Digestive Physiology and nutrition. Prentice Hall, New Jersey.

CHAPTER III

SUBACUTE RUMINAL ACIDOSIS IN STEERS IDENTIFIED AS HAVING CLINICAL BRD

Introduction

The incidence of bovine respiratory disease (BRD) continues to be high in feedlot cattle despite the use of preventive vaccines and other management and husbandry practices.[1] In 1999, most feedlots (97.4%) within 12 states reported an overall BRD incidence of 14.4%, nearly five times the percentage of the next most commonly reported disease, acute interstitial pneumonia.[2] The efficacy of vaccines in decreasing BRD morbidity and mortality is variable.[3,4,5] The variability is likely due to multiple factors such as prior vaccination, stress of commingling, transportation, environment, timing and inconsistent diets.[6]

High grain diets that are provided to backgrounding/receiving and feedlot cattle may play a significant role in increasing BRD morbidity and mortality.[7] Cattle that were fed soluble carbohydrate receiving diets had higher morbidity for BRD compared to cattle fed forage based diets.[7,8] The investigators speculated that high grain diets were probably causing some immunosuppression that resulted in animals being more susceptible to bovine respiratory disease pathogens, but the mechanisms of immunosuppression were not investigated.

Subacute ruminal acidosis (SARA) is a metabolic condition in ruminants, primarily cattle, caused by consumption of highly fermentable carbohydrates and characterized by repeated episodes of ruminal pH between 5.2 and 5.6.[9,10,11] Grain-induced SARA has been associated with increased free endotoxin in the rumen due to the death of gram negative bacteria[11,12] The increase in ruminal endotoxin could increase permeability of the gut for endotoxin.[13] Also, the barrier function of rumen epithelium may be compromised by the parakeratosis, rumenitis, and abscesses of the rumen wall that result from high rumen acidity.[14] Endotoxin in circulation is considered to be one of the factors that result in increased production of acute phase proteins, haptoglobulin and serum amyloid A (SAA) in cattle with SARA.[15] These increases in acute phase proteins, which are part of the acute phase response, indicate that SARA causes inflammation.[16] The SARA-induced inflammation could be local due to the damage to the gastrointestinal mucosa or systemic when there is absorption of immunogenic compounds.[16]

Repeated stimulation of the innate immune system by circulating endotoxin is thought to cause immunosuppression.[15] The mechanisms of immunosuppression have not been reported in cattle. Mouse and human monocytes and macrophages exposed to low levels of endotoxin (lipopolysaccharide) have been shown to have diminished pro-inflammatory response both in vitro and in vivo.[17,18,19,20] A condition in which cells that are exposed to low concentrations of endotoxin enter into a transient unresponsive state and are unable to respond to further challenges with endotoxin in the same magnitude is called endotoxin tolerance[18]. Mechanistically, endotoxin

tolerance is considered to be caused by dysregulation of inflammatory pathways.[17] Endotoxin tolerance has not been investigated or reported in cattle. There are currently no known reliable markers for both SARA and BRD.[21] In backgrounding/receiving and feedlot cattle, diagnosis of BRD often involves a subjective clinical assessment and measuring body (rectal) temperature.[21] In general, animals that appear sick but are not exhibiting clinical signs ascribable to any other body system are considered to have BRD[17,21]. Conditions that might indicate the problem of SARA in dairy herds include low body conditions, diarrhea, unexplained high cull rates due to vague health problems, low milk fat, and lower milk production in second and higher lactation cows compared to first lactation cows[22,23]. Dry matter intake also decreases in experimentally induced SARA in dairy cattle.[24] In dairy cattle, there is a clear distinction between SARA and acute ruminal acidosis which has specific clinical signs and often is fatal.[25] In contrast, generally there is no clear distinction between acute ruminal acidosis and SARA in beef cattle. The difficulty with separating acute ruminal acidosis and SARA may be caused by lack of diagnostic markers of SARA. In some situations, SARA may precede acute ruminal acidosis. The absence of ideal diagnostic markers for both SARA and BRD explain, at least in part, lack of concordance between treatment of clinical BRD and occurrence of lung lesions at slaughter.[26] Therefore, cattle experiencing SARA can potentially be misdiagnosed with BRD and treated with antimicrobials. The purpose of this study was to monitor rumial pH and endotoxin in cattle identified as having clinical BRD and also to measure outcomes in those animals through fifty six (56) day after arrival.

Materials and Methods

The study was approved the Oklahoma State University, Institutional Animal Care and Use Committee (IACUC). Recently weaned cattle from multiple sources that had been on grass pastures were purchased. Cattle were selected into the study if they were identified as exhibiting clinical signs of BRD. Thirty eight out of one hundred and forty six (26%) steers were sampled.

All animals were processed and managed according to the protocols at the Willard Sparks Beef Research Center, Oklahoma State University. Processing included dehorning, castration, deworming using injectable ivermectin[i] subcutaneously, vaccination using a modified live viral vaccine[ii] subcutaneously, and antimicrobial metaphylaxis using tilmycosin[iii] subcutaneously for the prevention of BRD in these high-risk cattle. Cattle that met the case definition for clinical BRD were treated with the second and third antimicrobials which were florfenicol[iv] and ceftiofur crystalline free acid[v] respectively. All medications were administered according to the label instructions. Cattle were fed a diet that met or exceeded the NRC guidelines. The diet contained cracked corn, corn gluten, and sorgum Sudan hay. In addition, minerals, vitamins, tylosin[vi] and monensin[vii] were added to the feed. Overall, the feed was 25% fiber and 75% concentrate per dry matter basis. The feed was provided twice a day at 07:00 and 13:00 as a total mixed ration. Fresh water was provided *ad libitum* through automatic watering stock tanks.

Footnotes : i-Ivermectin Plus®, ii- Pyramid® 5; iii-Micotil®; iv-Nuflor®; v-Excede®; vi-Tylan®; vii-Rumensin®

Clinical observation for BRD diagnosis

An experienced clinician observed the animals daily to identify clinical signs attributable to BRD or other diseases using the DART ™ system with some modifications.[27] Specifically, the subjective criteria used to indicate clinical BRD included depression (D), abnormal appetite (A), and respiratory signs(R). Signs of depression included depressed attitude, hanging head, glazed or sunken eyes, slow movement, arched back, difficulty getting up from lying down, knuckling or dragging toes when walking, and stumbling when moving. Signs of abnormal appetite included completely off feed, eating less than expected, slow eating, lack of fill (sunken left paralumbar fossa), and obvious body weight loss. Respiratory signs included obvious dyspnea (labored breathing), extended head and neck (orthopnea), and audible noise when breathing. The evaluator also assigned a severity score of 1 to 4, where 1 was assigned for mild signs, 2 for moderate signs, 3 for severe signs, and 4 for moribund (steer would not rise from recumbency; and/or assistance was needed to rise) during their evaluation. The fourth criterion used to determine if antimicrobial therapy was to be administered to an individual animal basis was an objective measurement of rectal temperature. Any animal with a rectal temperature of 104°F or greater received an antimicrobial according to label directions. In addition, any animal with a severity score of 3 or 4 was treated with an antimicrobial regardless of the rectal temperature. Any animal with severity score of 1 or 2 and a rectal temperature ≤ 103.9° F was not administered an antimicrobial. All cattle were returned to their home pen after their objective evaluation unless there was a concern for the well-being of the individual animal. Animals that were considered to require close monitoring were housed in special pens.

Rumenocentesis

Rumenocentesis was performed according to a previously described procedure in dairy cows with minor modifications.[28] The animal was restrained in a squeeze chute and tail jacking was performed. An area of about 10cm X 10cm located about 15 cm caudoventral to the

costochondral junction of the last rib on a line parallel with the proximal aspect of the stifle was identified. The area was clipped, scrubbed three times with a povidone-iodine scrub, and sprayed with 70% isopropyl alcohol. A 16 gauge, 4 inch stainless steel needle was inserted into the ventral sac of the rumen and a 10 mL syringe was used to aspirate a minimum of 2 mL of ruminal fluid. Ruminal pH was measured immediately using a calibrated hand-held digital pH meter (Horiba Instruments Incorporated, Irvine, CA). Rumen fluid samples were stored on ice until during transportation to the laboratory for processing. Processing of ruminal fluid samples included centrifugation at 10,000 × *g* for 45 minutes. The supernatant was aspirated gently to prevent its mixing with the pellet and passed through a disposable 0.22-μm LPS-free filter (Millex, Millipore Corporation, Bedford, MA). The filtrate was collected in a sterile, certified endotoxin free glass tube (Charles River, Charleston, SC) and heated at 100°C for 30 min. Samples were cooled at room temperature (23°C) for 10 min and stored at −20°C for subsequent endotoxin measurement.

Endotoxin testing

The concentration of LPS in plasma was determined by a kinetic chromogenic limulus amoebocyte lysate (LAL) assay (Charles River) with a minimum detection limit of 0.001 endotoxin units (EU)/ mL. All the materials that were used from sample collection to the measurement of endotoxin were certified endotoxin free. A 10-times serial dilution of the standard endotoxin (Escherichia coli 0111:B4) was prepared from a stock that contained 1000 (EU)/mL. The concentration of prepared standards ranged from 0.1 EU/mL to 1000 EU/ mL. Pretreated rumen samples were diluted in a serial manner until their endotoxin concentrations were in the range of standards. A 1000–times dilution was adequate for the majority of samples. Samples that were more concentrated than the highest concentration of standards were diluted further. The pH of the diluted ruminal fluid was determined to be neutral by measuring aliquots of the samples using a pH meter (Horiba Instruments Incorporated, Irvine, CA). All samples were tested in duplicate. A 96-well plate was used for the assay. 100 μL of each of the standards and

samples was added to the designated well on the plate. There were also wells for the blank, negative control and spiked ruminal fluid samples for validating non-interference. Only 200 µL of LAL water was added to the blank well. The negative control well contained 100 µL of LAL water and 100 µL of LAL reagent. Ten (10) µL of the 10 EU/mL endotoxin standard was added to the spike wells that contained 100 µL of ruminal fluid. A plate reader with a LAL software (Gen 5, Biotek) was used for the assay. The plate was incubated in the plate reader at 37° C for 10 minutes. The LAL reagent was dissolved in endotoxin-specific buffer to reduce interference. 100 µL of LAL was added to the all the wells excluding the blank. The plate was read at 405nm for 30 minutes. The software performed the analysis and provided the concentrations of samples based on the used endotoxin standards. The assay was considered valid if four conditions were met; that is, the was no contamination in the negative control, the regression curve generated by the standards was ≥ 0.98, the intra-assay coefficient of variation (CV) was ≤ 10 %, and the recovery from the spiked samples was between 50 and 200%.

Protozoa viability

A drop of ruminal fluid was put on a warm (37°C) microscope slide and covered with a cover slip. The slides were evaluated using a light microscope with a 10X objective lens. A combination of semi-quantitative and qualitative approach was used to evaluate protozoa viability. The decision to use this approach was made by the investigators because the the amount of ruminal fluid need for a comprehensive quantitative approach was insufficient. The designed semi-quantitative approach was considered to be easy and consistent from another research conducted the same investigators (unpublished data). Three categories were created to grade the protozoa viability. Grade 1 was assigned to protozoa that were few in numbers and had sluggish motility, grade 2 was for moderate numbers and fair to good motility, grade 3 was for overwhelming numbers and excellent motility.

Statistical analysis

SAS 9.3 was used for data analysis. A Wilcoxon rank sum test was used to compare body temperatures, pH values, and average daily gain between the treated and the untreated group. A Kendall's tau was used to evaluate protozoa viability between the two groups. Descriptive statistics were used categorical information.

Results

Sixty seven (67) out of one hundred and forty six (146) steers (46%) were pulled for clinical BRD during the study period. Thirty eight (38) of the pulled animals were randomly selected for the study. Twenty (20) out of thirty eight (38) steers met the criteria for clinical BRD treatment with antimicrobials (Treated group). Eighteen (18) out of thirty eight (38) sampled steers did not have a rectal temperature ≥ 104° F or severity score > 2 and were not treated for BRD (non-treated group). Eleven (11) out the eighteen (18) steers (61%) in the non-treated group had SARA (ruminal pH≤ 5.6). Two out of the twenty steers (10%) in the treated group had SARA. Five (5) steers from the non-treated group had been pulled but did not meet the treatment criteria prior to the first sample collection. Ten (10) steers from the treated group were pulled more than once; one out of the ten had SARA, three had normal ruminal pH and no samples were obtained from the other six because they were not selected during the random selection method. Four (4) out of eleven steers that had SARA at first sampling were subsequently pulled three times but did not meet the treatment criteria on all occasions. Two (2) out of the eighteen steers in the non-treated group were pulled four times but did not meet the treatment criteria. One animal was pulled seven times; three of the pulls were on consecutive days but did not meet the treatment criteria on all occasions. Fifty-one out of sixty seven (76%) were pulled during the first thirty days. Ten out of 38 sampled steers died. All dead animals had bronchopneumonia that was confirmed by necropsy. Two of the dead steers had ruminal pH values below 5.6 at the time of sampling.

The median pH value for the non- treated was 5.59, while those treated had a median pH of 6.6. There was a significant difference in pH between the treated and the not-treated groups ($p<0.0005$). There was no statistical difference in the average daily gain (ADG) distribution between the treated group and the non-treated group ($p=0.44$). Mean ADG was 2.2 pounds and 1.9 pounds for the non-treated and the treated groups respectively. For protozoa viability, there was a significant association ($p<0.005$), with treated calves having a lower protozoa viability score than those that were not treated.

The median amount of endotoxin in the non-treated and the treated was 39 870 EU/mL and 56 723 EU/mL. There was no statistical difference in the median amount of endotoxin in the ruminal fluid between the treated and non-treated group ($p=0.184$). No correlations were found between endotoxin levels and pH ($p=0.24$), ADG ($p=0.29$) or rectal temperature ($p=0.4$).

Discussion

The most significant finding from the current study was the difference in ruminal pH between steers that were identified for clinical BRD and met the antimicrobial treatment criteria and those identified but failed to meet the treatment criteria. It has been observed that at Willard Sparks Beef Research Center, there are always some animals that do not exhibit clinical signs ascribable to any particular disease but appear different to an experienced observer (Step & Krehbiel, personal communication). Animals that have such non-specific signs are presumed to have clinical BRD and are therefore treated with antimicrobials if they meet the treatment criteria of body temperature greater than or equal to104°F or a clinical severity score of 3 or 4.[21,27] This current study suggest that beef cattle that are fed receiving diets, and have nonspecific clinical signs of disease but fail to meet the criteria (rectal temperature) for antimicrobial treatment, might be suffering from subcute ruminal acidosis.

Eleven out of eighteen steers (61%) in the group that did not get antimicrobial treatment had SARA that was defined by ruminal pH ≤ 5.6.[12] A similar study at feedlot facility did not find a difference in ruminal pH between exhibiting rectal temperatures of ≥ 104° F or <104° F.[29] In that study, cattle exhibiting temperatures ≥ 104° F had an average ruminal pH of 5.95 whereas, cattle exhibiting rectal temperatures < 104° F had an average ruminal pH of 6.03. In the current study, higher rectal temperatures were associated with higher ruminal pH ($p< 0.0005$). The reasons for difference between the two studies are not clear but it could be due to the differences in personnel who identified the morbid cattle, the time at which rumenocentesis was performed or some unidentified difference. Differences in equipment might also lead to different accuracy of measurements that might affect the ability to detect small differences. The ability to identify cattle with very subtle clinical signs could mean that morbid cattle are identified during the early course of the disease and hence the detection of differences in ruminal pH.

Interestingly, ten of the steers from the non -treated group (55%) continued to be morbid although they never satisfied the requirements for antimicrobial therapy. There was one steer that was pulled seven times during the first 36 days but was never treated with antimicrobials. The ruminal pH of the steer that was pulled seven times was 5.52 and 5.69 on the two occasions that rumenocentesis was performed. Rumenocentesis was not performed on the same steers the other 5 times because he was pulled outside the regular sampling time and the investigators were unavailable. These findings suggest that SARA is not a benign condition and further research into the pathophysiology and resulting complications is necessary. Acute ruminal acidosis and SARA are two separate conditions although SARA might precede the former. Research should also focus on evaluating measures that prevent acidosis in beef cattle.

Interestingly, there was no statistical difference in the average daily gain for the two groups. In a sixty day preconditioning period, cattle that were slow to gain weight at the beginning might be able to compensate such that the overall weight gain might not be statistically different.[27]

Protozoa viability was significantly higher in the non-treated group. The information on protozoa viability in SARA in the literature is unclear.[30] It is suggested that the viability of protozoa initially decreases due to low ruminal pH but protozoa thrive at a pH of 5.5 in the rumen of limit-fed cattle consuming high-starch purified diets.[30] Protozoa are affected by the pH and intake and are therefore expected to be low in overtly diseased animals with anorexia.

The amount of endotoxin in the ruminal fluid of pulls animals was not related to pH, temperature or average daily gain. Both groups of animals had some morbidity which likely affects digestion in the rumen and hence changes in the levels of endotoxin. The absence of controls (not pulled but sampled) limits the interpretation of endotoxin levels.

Conclusion

A significant number of steers that were identified as having signs of clinical BRD had SARA. This study suggests that the occurrence of SARA in cattle that are on backgrounding or receiving diets might complicate the diagnosis of clinical BRD. Further research is necessary to understand the interaction between BRD and SARA.

References

1. Taylor JD, Fulton RW, Lehenbauer TW, Step DL, Confer AW. The epidemiology of bovine respiratory disease: What is the evidence for preventive measures? *Can Vet J* 2010; 51(12): 1351–1359.

2. NAHMS. 2000a. Feedlot '99 Part II: Baseline Reference of Feedlot Health and Health Management. USDA, APHIS, National Animal Health Monitoring System. Available: http://www.aphis.usda.gov/animal_health/nahms/feedlot/downloads/feedlot99/Feedlot99_dr_PartII.pdf. Accessed August 24, 2014.

3. PerinoLJ, Hunsaker BD. A review of bovine respiratory disease field efficacy. *Bov Pract* 1997: 59-66.

4. Larson RL, Step DL. Evidence-based effectiveness of vaccination against Mannheimia haemolytica, Pasteurella multocida, and Histophilus somni in feedlot cattle for mitigating the incidence and effect of bovine respiratory disease complex. *Vet Clin North Am Food Anim Pract* 2012; 28:97-106,106e101-107, ix.

5. Morter RL, Amstutz HE. Evaluating the efficacy of a *Haemophilus somnus* bacterin in a controlled filed trial. *Bov Pract* 1983; 18:82-83.

6. Tripp HM, Step DL, Krehbiel CR; Moberly HK, Malayer JR. Evaluation of outcomes in beef cattle comparing preventive health protocols utilizing viral respiratory vaccines. *Bov Pract* 2013; 47(1):54-64.

7. Lofgreen GP. Nutrition and management of stressed beef calves. *Vet Clin North Am Food Anim Pract* 1983; 5:87-101.

8. Mwansa PB, Makarechian M, Berg RT. The effect of level of concentrate in feedlot diets on the health status of beef calves. *Can Vet J* 1992; 33: 665-668.

9. Cooper R, Klopfenstein T. 2006. Effects of Rumensin and feed intake variation on ruminal pH. Update on Rumensin / Tylan /Micotyl for the Professional Feedlot Consultant. Elanco Animal Health, Greenfield, IN.

10. Gozho GN, Plaizier JC, Krause DO, Kennedy AD, Wittenberg KM. Subacute Ruminal Acidosis Induces Ruminal Lipopolysaccharide Endotoxin Release and Triggers an Inflammatory Response. *J. Dairy Sci*.2005; 88:1399–1403.

11. Nagaraja TG,Titgemeyer EC. Ruminal acidosis in beef cattle: The current microbial and nutritional outlook.J. Dairy.SCi (E Suupl) 2007; 90:E17-E38

12. Khafipour E, Krause DO, Plaizier JC. A grain-based subacute ruminal acidosis challenge causes translocation of lipopolysaccharide and triggers inflammation. *J Dairy Sci 2009*; 92:1060-1070.

13. Chin AC, Flynn AN, Fedwick JP, Buret AG. The role of caspase-3 in lipopolysaccharide-mediated disruption of intestinal epithelial tight junctions. *Can. J. Physiol. Pharmacol* 2006; 84:1043–1050

14. Kleen JL, Hooijer GA, Rehage J, Noordhuizen JP. Subacute ruminal acidosis (SARA): A review. J. Vet. Med. *A Physiol. Pathol. Clin. Med* 2003; 50:406–414

15. Zebeli Q, Metzler-Zebeli BU. Interplay between rumen digestive disorders and diet-induced inflammation in dairy cattle. *Research in Veterinary Science* 2012; 93:1099–1108.

16. Horadagoda NU, Knox KM, Gibbs HA, Reid SW, Horadagoda A, Edwards SE, Eckersall PD. Acute phase proteins in cattle: Discrimination between acute and chronic inflammation. *Vet. Rec* 1999; 144:437–441.

17. Zuckerman SH, Evans GF, Snyder YM, Roeder WD. Endotoxin-macrophage interaction: post-translational regulation of tumor necrosis factor expression. *J. Immunology* 1989; 143(4): 1223-1227.

18. Mengozzi M, Ghezzi P. Cytokine down-regulation in endotoxin tolerance. *Eur. Cytokine Netw* 1993; 4 (2): 89–98.

19. Takasuka N, Tokunaga T, Akagawa KS Preexposure of macrophages to low doses of lipopolysaccharide inhibits the expression of tumor necrosis factor-alpha MRNA but not of IL-1 beta MRNA. *J. Immunol* 1991; 146 (11): 3824–3830.

20. Medvedev AE, Kopydlowski KM, Vogel SN. Inhibition oflipopolysaccharide-induced signal transduction in endotoxin-tolerized mouse macrophages: dysregulation of cytokine, chemokine, and toll-like receptor 2 and 4 gene expression. *J. Immunol* 2000; 164 (11): 5564–5574.

21. Leruste H, Brscic M, Heutinck LF, et al. The relationship between clinical signs of respiratory system disorders and lung lesions at slaughter in veal calves. *Prev Vet Med* 2012; 105: 93–100.

22. Oetzel GR, Monitoring and testing dairy herds for metabolic disease. *Vet Clin Nor Amer Food Anim Pract* 2004; 20: 651–674.

23. Colman E, Fokkink WB, Craninx M, Newbold JR, De Baets B, Fievez V. Effect of induction of subacute ruminal acidosis on milk fat profile and rumen parameters. *J Dairy Sci* 2010; 93: 4759-4773.

24. Brown MS, Krehbiel CR, Galyean ML, Remmenga MD, Peters JP, Hibbard B, Robinson J, Moseley WM. Evaluation of models of acute and subacute acidosis on dry matter intake, ruminal fermentation, blood chemistry, and endocrine profiles of beef steers. *J Anim Sci* 2000; 78:3155-3168.

25. Underwood WJ. Rumen Lactic Acidosis. Part II. Clinical signs, Diagnosis, Treatment, and Prevention. *Compendium* 1992; 9(8): 1265-1270.

26. Wittum TE, Wollen NE, Perino LJ, Littledike TE. Relationships among treatment of respiratory tract disease, pulmonary lesions evident at slaughter and rate of weight gain in feedlot cattle. *J Am Vet Med Assoc*. 1996; 209:814-818.

27. Step DL, Krehbiel CR, Depra HA et al. The effects of commingling beef calves from different sources and weaning protocols during a forty- two -day receiving period on performance and bovine respiratory disease. *J Anim Sci* 2008, 86:3146-3158.

28. Garret EF, Pereira MN, Nordlund KV, et al Methods for the Detection of Subacute Ruminal Acidosis in Dairy Cows. *J Dairy Sci* 1999; 82:1170–1178.

29. Thomson DU. Monitoring of rumen pH in cattle pulled for bovine respiratory disease complex with normal or elevated rectal temperatures in a commercial feedlot. In Proceedings of the Fortieth Annual Conference, American Association of Bovine Practitioners, Vancouver, British Columbia, Canada, 20-22 September, 2007. AABP 273.

30. Owens FN, Goetsch AL. 1988 .Ruminal Fermentaion in The ruminant animal: Digestive Physiology and nutrition. Prentice Hall, New Jersey.

CHAPTER IV

INNATE IMMUNE RESPONSE TO SUBACUTE RUMINAL ACIDOSIS IN BEEF CATTLE

Introduction

Subacute ruminal acidosis (SARA) is a metabolic condition caused by consumption of highly fermentable carbohydrates and characterized by ruminal pH between 5.2 and 5.6 for at least 3 hours.[1,2,3] Grain-based subacute ruminal acidosis (SARA) has been associated with increases in circulating acute phase proteins haptoglobulin and serum amyloid A.[4] The increase in circulating acute phase proteins suggests that SARA causes inflammation.[5] The inflammation may be caused by dietary-induced damage to the gastrointestinal mucosa or by translocation of pathogen associated molecular patterns (PAMPs), alarmins or chemical compounds into systemic circulation (Horadagoda 1999).[5]

Endotoxin (lipopolysaccharide, LPS) is one of the PAMPs that have been shown to be elevated in the rumen and blood of cattle with grain-induced SARA..[3] Increased proliferation and lysis of gram-negative bacteria result in elevated levels of endotoxin in the rumen.[3,6] Decreased pH compromises the natural barrier function of the rumen epithelium by rumenitis, parakeratosis and abscessation.[7] Additionally, the high ruminal osmolality that is seen during SARA can cause swelling and rupture of ruminal papillae, which will also reduce the barrier function of the rumen.[8] Various genes of the stratified squamous epithelium of the rumen were also down-regulated during adaption to a 65% concentrate diet suggesting that there are molecular as well as morphologic changes when high grain diets are provided.[9]

The development of SARA causes changes in the composition of microbes as well as digestion in the rumen. Decreases in ruminal pH in animals experiencing SARA is also accompanied by increases in total volatile acid production and also shifts in microbial populations.[10,11] SARA is characterized by decreases in acetate: propionate ratio and normal levels of butyrate because the lower ruminal pH favors the growth of amylolytic and lactate- utilizing bacteria whereas the growth of cellulolytic bacteria is inhibited.[10,12] Studies have demonstrated a shift of ruminal microbes from a predominantly gram negative bacteria to a predominantly gram positive bacteria in animals experiencing SARA.[3,10,11] Another study has also demonstrated marked increases in *Eschericia coli* strains and their virulence factors in ruminal fluid of animals experiencing SARA.[13] The investigators speculated that the observed increases in acute phase proteins were a result of action of increased levels of virulent *E. coli* acting locally causing inflammation in circulation.[13] The diversity of ruminal bacteria also decreases when animals are receiving step-up high grain diets.[10]

The concentration of biogenic amines also increases in the rumen of cattle that are being fed fermentable carbohydrates. Biogenic amines are low molecular weight organic bases present in all organisms. The most common forms are tyramine, putrescine, histamine, methylamine and

tryptamine. At low concentrations, they are essential for normal growth and differentiation of cells[14], but in larger quantities (1.4 g per day) they become harmful to humans and livestock.[14] Histidine and other amino acids normally undergo deamination in the rumen, whereas decarboxylation to form biogenic amines including histamine, putrescine, tyramine, methylamine and tryptamine does not normally occur in the rumen to any great extent[15]. However, when sheep or cattle are fed excessive amounts of rapidly fermentable carbohydrates, there can be an increase in the content of histamine or other biogenic amines in the ruminal fluid.[15] The relationship between ruminal fluid biogenic amines and biogenic amines in blood is an area of active research.[15] The mechanism of toxicity of biogenic amines in cattle is not known.

Clinical signs of SARA are nonspecific. Animals with SARA can have decreased dry matter intake, decreased milk production, increased incidence of laminitis and related feet problems, soft cloudy feces and, decreased ruminal contractions.[16] Cattle that were receiving high grain diets had higher morbidity for bovine respiratory disease compared to cattle receiving forage diets.[17,18] The investigators speculated that high grain diets caused some immunosuppression that resulted in animals becoming more susceptible to bovine respiratory disease pathogens. In another study, two groups of mice were challenged with oral and intratracheal endotoxin. The animals that received oral endotoxin developed pulmonary edema and had higher concentrations of mRNA of inflammatory cytokines in systemic circulation than in bronchoalveolar lavage fluid (BALF).[19] In contrast, animals that received intratracheal endotoxin had higher concentrations mRNA of inflammatory cytokines in the BALF than in systemic circulation. This study suggests that endotoxin is absorbed from the gastrointestinal system into the systemic circulation, and causes inflammation and acute lung injury. However more cytokines are produced when the insult is coming directly from the trachea than from systemic circulation.

The objectives of this study were to investigate ruminal pH, ruminal temperature, ruminal microbial populations, ruminal endotoxin, and ruminal biogenic amines in steers receiving

varying amounts of fermentable carbohydrates in their diets. Correlations were also made between ruminal fluid endotoxin and plasma endotoxin concentration. Correlations were also made between fold change of cytokine expression in BALF and blood.

Materials and methods

The study was approved the Oklahoma State University, Institutional Animal Care and Use Committee (IACUC). Thirty two (32) animals were randomly selected from a group of 400 commingled recently weaned calves from multiple sources in Oklahoma. The animals were on native grass pastures prior to purchase and enrollment into the study. The animals were randomly assigned into each of the four groups; Control, 40%CHO, 60%CHO and 80%CHO. The control group received a 20% concentrate: 80% forage diet; the 40%CHO received a 40% concentrate: 60% forage diet; the 60%CHO received a 60% concentrate: 40% forage; the 80%CHO group received an 80%: concentrate 20% forage per dry matter basis. In addition, minerals, vitamins, tylosin[i] and monensin[ii] were added to the feed. Animals were fed two times a day, at 07:00 and at 17:00. Water was provided *ad libitum* via an automatic waterer. All animals were processed and managed according to the protocols at the Willard Sparks Beef Research Center, Oklahoma State University. Processing included dehorning, castration, deworming using injectable ivermectin[iii] subcutaneously, vaccination using a modified live viral respiratory vaccine[iv] subcutaneously, and antimicrobial metaphylaxis using tilmycosin[v] subcutaneously for the prevention of BRD in these high-risk cattle. Cattle were fed a diet that met or exceeded the NRC guidelines. The diet contained cracked corn, corn gluten, and Prairie hay. The proportions of the dietary constituents varied according to the amount of highly digestible/fermentable carbohydrates.

Footnotes: i-Tylan®, ii-Rumensin®, iii-Ivermectin plus®, iv-Bovishield Gold®, v-Micotil®,

Rumenocentesis

Rumenocentesis was performed according to a previously described procedure in dairy cows with minor modifications.[20] The procedure was performed weekly for four weeks. The first samples (baseline) were collected on the day of processing. The animal was restrained in a squeeze chute and tail jacking was performed. The person performing the rumenocentesis identified a 10cm X 10cm area located approximately 15 cm caudoventral to the costochondral junction of the last (13^{th}) rib on a line parallel with the most proximal aspect of the stifle. The area was clipped, scrubbed with a povidone-iodine scrub, and sprayed with 70% isopropyl alcohol. A 16 gauge, 4 inch stainless steel needle was inserted into the ventral sac of the rumen and a 10 mL syringe was used to aspirate a minimum of 2 mL of ruminal fluid. Ruminal pH was measured immediately using a hand-held digital pH meter (Horiba Instruments Incorporated, Irvine, CA). Ruminal fluid samples were transferred to two separate vials. One of the vials was certified nonpyrogenic (Corning external thread cryogenic vials, Corning, NY). Both sets of tubes were stored on ice during transportation. Aliquotes of 200µL of ruminal fluid were transferred from the clean (non-sterile) tube and stored at -80° C until were used in the isolation of DNA for qRT-PCR. The nonpyrogenic vials were centrifuged at 5 000 x g for 15 minutes. The supernatant was filtered by a 0.2 micron syringe filter (Millipore). The fluid was filtered into two separate tubes; a certified endotoxin free tube (Charles River) for endotoxin measurement and a clean vial for high performance liquid chromatography (HPLC). All the samples were stored at -80° C until they were analyzed.

Clinical observation for BRD diagnosis

An experienced clinician observed the animals daily to identify clinical signs attributable to BRD or other diseases using the DART ™ system with some modifications.[21] Specifically, the subjective criteria used to indicate clinical BRD included depression (D), abnormal appetite (A),

and respiratory signs (R). Signs of depression included depressed attitude, hanging head, glazed or sunken eyes, slow movement, arched back, difficulty getting up from lying down, knuckling or dragging toes when walking, and stumbling when moving. Signs of abnormal appetite included completely off feed, eating less than expected, slow eating, lack of fill (sunken left paralumbar fossa), and obvious body weight loss. Respiratory signs included obvious dyspnea (labored breathing), extended head and neck (orthopnea), and audible noise when breathing. The evaluator would also assign a severity score of 1 to 4, where 1 was assigned for mild, 2 for moderate, 3 for severe, and 4 for moribund (animal would not rise from recumbency; assistance was needed) during their evaluation. The fourth criterion used to determine if antimicrobial therapy was needed on an individual animal basis would be an objective measurement of rectal temperature. Any animal with a rectal temperature of 40°C or greater would receive an antimicrobial according to label directions. Any animal with a severity score of 3 or 4 would also be treated with an antimicrobial regardless of the rectal temperature. Any animal diagnosed with a disease other than BRD would be treated as directed by an experienced clinician.

In addition, ruminal boluses were also administered to monitor ruminal temperature. The boluses transmitted signals to the central processor continuously. Data was then retrieved and analyzed by statistical software (SAS 9.3).

Bronchoalveolar lavage fluid (BALF) collection

BALF was performed weekly for four weeks. Bronchoalveolar lavage fluid was collected according to a previously described procedure with some minor modifications.[22] Briefly, heifers were restrained in a squeeze chute and cross ties were used to position the head so that the heifer's nose was elevated. Then, samples were obtained by inserting a sterile 240 cm-long BAL tube (Broncho-alveolar lavage equine catheter J639, Jorgensen Laboratories, Loveland, CO) equipped with a three-way stop cock into one of the nares. The BAL tube was passed through the

trachea, past the tracheal bifurcation, into a distal lung lobe, and the area was sealed by inflating the cuff with approximately 10 mL of air. A 60 mL syringe containing sterile phosphate buffered saline (PBS) solution was attached to the stopcock, which was then opened to allow instillation of PBS. Solution was immediately aspirated, and retrieval was typically 50 – 75% of the amount instilled. The samples were placed in a cooler with ice and transported to the laboratory. RNA was isolated on the same day. If the tube was inadvertently advanced into the esophagus, it was withdrawn and a different sterile tube was used. BALF was stored on ice and RNA was isolated on the same day.

DNA isolation and quantitative real time PCR

DNA was isolated from ruminal fluid samples using a DNA isolation kit (QIAamp Stool Mini Kit, Qiagen) and following the manufacturer's protocol. Briefly, lysis buffer was added to the 200uL samples that had been frozen. The mixture was incubated at 70° C for 5 minutes. Samples were centrifuged and an InhibitEX tablet was added to each sample to bind inhibitors. Proteinase K, buffer and ethanol were added and samples were incubated at 70° C for 10 minutes. Samples were loaded into spin columns and washed twice with separate solutions before DNA was eluted with 100 uL of elution buffer. The isolated DNA was stored at -20° C until further analysis. Quantitative real time PCR was performed to quantify specific bacteria based on their unique 16S DNA sequences. Quantitative analysis of population changes of selected bacterial species were conducted using real-time polymerase chain reaction (qRT-PCR). Ten animals were randomly selected from the animals that had at least one recorded ruminal acidosis episode, pH≤ 5.6 and another ten were randomly selected from the animals that had never had a recorded acidosis episode. The selected bacteria included *Prevotella ruminocola*, *Ruminococcus flavifaciens*, *Fibrobacter succinogenes*, *Megasphaera elsdenii*, *Streptococcus bovis*, and *Lactobacillus acidophilus*.

Each qPCR assay was performed using a SYBR green assay kit (Roche Diagnostics, Indianapolis, IN). The dynamic range and PCR efficiency of each assay were evaluated using positive controls. A 15µl reaction mixture contained 5 uM (each) forward and reverse primer, 7.5µl Sybr Green Master mixture (Roche Diagnostics), and 30 ng of ruminal fluid DNA. Thermal cycling conditions were 95°C for 10 min, followed by 50 additional cycles of 95°C for 30 s, 54°C for 30 s, and 72°C for 40 s or 1 min, and finally, a melting curve was prepared. A universally conserved fragment of 16S was used for normalization. The qPCRs were performed using a MyIQ RT-PCR instrument (BioRad). Agarose gel electrophoresis was used to verify amplification of the correct products. The primers and PCR conditions are shown in Table 4. Relative quantification of bacterial population changes was performed using the comparative threshold cycle (*CT*) method, as described previously.[10]

Bacteria	Primer sequence 5'-3'	Product size (bp)	Annealing Temp (° C)
Prevotella ruminocola	F-GGTTATCTTGAGTGAGTT R-CTGATGGCAACTAAAGAA	485	54
Megasphaera elsdenii	F-GACCGAAACTGCGATGCTAGA R-TCCAGAAAGCCGCTTTCGCCACT	128	54
Streptococcus bovis	F-ATTCTTAGAGATAGGGTTTCTCTT R-ACCTTATGATGGCAACTAACAATA	134	54
Lactobacillus acidophilus	F-GTTCCTTCGGGGACACTAAGACAG R-TCCCGAGTTAGGCCACCGGCTTTG	450	54
Fibrobacter succinogenes	F-GGTATGGGATGAGCTTGC R-GCCTGCCCCTGAACTATC	445	54
Ruminococcus flavefaciens	F-GGACGATAATGACGGTACTT R-GCAATCYGAACTGGGACAAT	295	54
Universal 16S	F-AGACTTTGATCCTGGCTCAG R-TGCTGCCTCCCGTAGGAGT	805	54

Table. 4. Primer sequences used for quantitative real time of specific 16S gene of various ruminal bacteria.

RNA isolation and qRT- PCR

The expression of mRNA of multiple cytokines was determined by real time RT-PCR. RNA isolation was performed on the same day the samples were obtained. Total RNA was extracted using an RNA extraction kit (High Pure RNA Isolation Kit, Roche) according to the manufacturer's protocol. Briefly, BALF was centrifuged at 2500 x g for 5 minutes to pellet the cells and the supernatant was discarded. Cells were resuspended in PBS and a lysis buffer was added. Samples were then transferred into filter tubes and centrifuged at 8000 x g for 15 seconds. Buffered DNAse was added to the tubes and incubated at room temperature (23° C) for 15 minutes. Tubes were twice with different solution before elution of RNA. RNA isolation from blood was similar to RNA isolation from BALF but there were two extra steps at the beginning. A red blood cell (RBC) lysis buffer was initially added to blood samples. After lysis of RBCs, the remaining leukocytes were washed twice with PBS. After resuspension of the leukocytes, procedure was similar to RNA isolation from BALF. The concentration of RNA was measured using spectrophotometry (Nanodrop, Thermo Scientific). RNA was stored at -80° C until further analysis. Copied DNA (cDNA) was generated using a reverse transcriptase kit (QuantiTect Rev. Transcription Kit, Qiagen). Briefly, RNA was mixed with RNAse-free water and DNA wipe-out buffer. The mixture was incubated at 42° C for 2 minutes. Reverse transcriptase, reverse transcription buffer and reverse transcription primer mix were added. The mixture was incubated at 42° C for 30 minutes. The tubes were then incubated at 95° C for 3 minutes to stop reverse transcription. The cDNA was subsequently used for real time PCR. PCR amplification and detection were performed in MyIQ, Biorad Detection System using the following cycling conditions: 95°C for 5 min and 40 cycles of 94° C for 15 s, 55° C for 30 s, and 72°C for 30 s. Final extension was at 72° C for 4 minutes. All RT-PCRs were carried out in duplicate with appropriate controls on each plate. Bovine b-actin mRNA was used for internalization. The

sequences of primers used are shown in Table 5. The fold change in cytokine expression was calculated using the comparative CT method as previously described.[23]

Cytokine	Sequence 5'-3'	Annealing Temp(°C)	Product size(bp)	Accession number
Bovine actin	F- CGC ACC ACT GGC ATT GTC AT R- TCC AAG GCG ACG TAG CAG AG	55	227	NM_173979.3
IL-1β	F- GAT GCC TGA GAC ACC CAA R- GAA AGT CAG TGA TCG AGG G	55	173	NM_174092.1
IL-8	F- CAC TGT GAA AAA TTC AGA AAT CAT TGT TA R- CTT CAC CAA ATA CCT GCA CAA CCT TC	55	107	NM_173925.2
IL-10	F- TGC TGG ATG ACT TTA AGG G R- AGG GCA GAA AGC GAT GAC A	55	186	NM_174088.1
TNF-α	F- TCT TCT CAA GCC TCA AGT AAC AAG T R- CCA TGA GGG CAT TGG CAT AC	55	103	NM_173966.3
TGFβ1	F- CTG AGC CAG AGG CGG ACT AC R- TTG CTG AGG TAG CGC CAG GAA TTG	55	262	NM_001166068.1

Table.5. Primer sequences for bovine cytokine genes that were used in qRT-PCR.

Endotoxin testing

The concentration of LPS in ruminal fluid and plasma was determined by a kinetic chromogenic limulus amoebocyte lysate (LAL) assay (Charles River) with a minimum detection limit of 0.001 endotoxin units (EU)/ mL. All the materials that were used from sample collection to the measurement of endotoxin were certified endotoxin free. A 10-times serial dilution of the standard endotoxin (*Escherichia coli* 0111:B4) was prepared from a stock that contained 1000 endotoxin units (EU)/mL. The concentration of prepared standards ranged from 0.1 EU/mL to 1000 EU/ mL for ruminal fluid. The concentration of prepared standards ranged from 0.001 EL/mL to 1000 EU/mL for plasma. Pretreated ruminal fluid samples were diluted in a serial manner until their endotoxin concentrations were in the range of standards. A 1:10 000 dilution was adequate for all the samples. Plasma samples were diluted 1:40. The pH of the diluted ruminal fluid was determined to be neutral by measuring aliquots of the samples using a pH meter (Horiba Instruments Incorporated, Irvine, CA). All samples were tested in duplicate. A 96-well plate was used for the assay. 100 µL of each of the standards and samples was added to the designated well on the plate. There were also wells for the blank, negative control and spiked ruminal fluid samples for validating non-interference. Only 200 µL of LAL water was added to the blank well. The negative control wells contained 100 µL of LAL water and 100 µL of LAL reagent. Ten (10) µL of the 10 EU/mL endotoxin standard was added to the spike wells that contained 100 µL of ruminal fluid. A plate reader with a LAL software (Gen 5, Biotek) was used for the assay. The plate was incubated in the plate reader at 37° C for 10 minutes. The LAL reagent was dissolved in endotoxin-specific buffer (carboxymethylated curdlan) to reduce interference. 100 µL of LAL was added to the all the wells excluding the blank. The plate was read at 405nm for 30 minutes. The software performed the analysis and provided the concentrations of samples based on the used endotoxin standards. The assay was considered valid if four conditions were met; that is, the was no contamination in the negative control, the

regression curve generated by the standards had $R^2 \geq 0.98$, the intra-assay coefficient of variation (CV) was ≤ 10 %, and the recovery from the spiked samples was between 50 and 200%.

Biogenic amines measurement

Chemicals

Methylamine hydrochloride, putrescine dihydrochloride, cadaverine dihydrochloride, piperidine, tyramine, heptylamine (internal standard) dansylchloride, and sodium tetraborate were obtained from Sigma –Aldrich, St Louis, MO. Acetonitrile, methanol, acetone and ethylacetate were obtained from Fisher Scientific, Pittsburgh, PA.

Sample preparation

Standards were initially prepared by weighing each standard and diluting with 2:1 acetonitrile to make a final concentration of 1mg/mL. The range of concentration of the standard calibration curve was 0.1 µg/mL to 100 µg/mL. The standard stock solutions were prepared by serial dilutions. The prepared stocks of standards were stored at -80° C until they were used. One hundred microliters (100 µL) of ruminal fluid or external standard solutions were added to labelled test tubes. Four hundred microliters (400 uL) of 2: 1 acetone water containing 5ug/mL of heptylamine (internal standard) was added. Borax buffer (100 µL-3.81g sodium tetraborate in 100mL HPLC water to pH 10.5 with 10 M sodium hydroxide), 1% dansylchloride (200 µL), and 2:1 acetone: water was added to make a final volume of 1 mL. The samples were vortexed for 10 minutes before they were incubated at 65° C in the dark for 25 minutes for derivatization to take place.

Solid phase extraction was carried out in SEP-PAK C 18, 3mL columns (Water, Milwaukee, WI). Columns were activated by adding 1 mL of methanol followed by 1 mL of HPLC water. One milliliter (1 mL) of the sample, blank, calibration standards or quality control samples were

loaded to the column. The flow rate was set at 1 drop / second. The columns were washed with 1 mL of 50% methanol. The derivatized amines were eluted in with ethylacetate. The samples were dried in Turbo Vap LV evaporator (Gen Tech Scientific) using nitrogen gas at 15-20 psi. The analytes were redisolved in a 1 mL mixture of mobile phase A (700 µL of 5% acetonitrile) and mobile phase B (300 µL of 100% acetonitrile). Analytes were then transferred to auto-sampler vials.

HPLC analysis

Chromatography was performed using a Varian Prostar liquid chromatogaph (Varian, Walnut Creek, CA) using the fluorescence detector. Separation was carried out using a Waters Symmetry Shield RP_{18} column (4.6 i.d. x 250mm, particle size 5 um). Compounds were detected by measuring fluorescence. The fluorescence excitation and emission wavelengths were 337, and 492 nm, respectively, and the photomultiplier tube (PMT) gain was 15.

Absolute acetonitrile was mobile phase B and 5% acetonitrile was mobile phase A. The flow rate was 1 mL/min. The following gradient was used for the separation: Time =0, 62% A and 38% B; time = 4 min, 62% A and 38% B; time=32 min, 20% A and 80% B; time= 32.5 min, 10% A and 90% B; time= 35 min, 62% A and 38% B. The run time was 45 min. Injection volume was 15 µL for the samples, standards and controls. Isopropanol (50 µL) was injected after every eight sample runs to clean the column because there was no guard column.

HPLC assay validation

Derivatized amines in ruminal fluid were identified according to their retention times compared standards obtained from pure amines. Standard curves were constructed for each amine and were linear between 0.1 and 100 µg/mL. Unknown concentrations were therefore calculated from the regression equation. The recovery of each amine was calculated by dividing the peak area of each solid-phase extracted amine by the peak area of the amine that did not go through solid phase

extraction. Stability of the amines was also calculated by dividing peak areas of the sample immediately after extraction by the peak area of the same sample when run after hours, 10 hours, 20 hours and 48 hours. The intra-assay coefficient of variation (CV) was calculated by the machine.

Statistical Analyses

Fold change data for ruminal bacteria and cytokine expression was performed with statistical software (SPSS). Multivariate analyses were performed to compare fold changes among the four groups on days 7, 14 and 21. Pearson's correlations were performed to determine relationship among the measured variables. ANOVA for repeated measures was used to analyze changes in endotoxin levels in both blood and ruminal fluid over time.

Results

Ruminal pH

The ruminal pH on a given day was significantly associated with the diet for each day examined. The day 7 pH association with diet was significant at P=0.001. Post-hoc analysis found the only significant association on pair-wise comparisons to be the control group having higher pH than did the 80%CHO group (P=0.001). No other differences were significant.

Day 14 pH was significantly associated with diet group (P<0.0005). The only significant pair-wise comparison was the control group having a higher pH than the 80%CHO group. The 40%CHO group was trending toward being significantly different from the 80%CHO (P=0.057). Day 21 pH was significantly associated with diet (P<0.0005). Post-hoc comparisons found a significantly higher pH in controls vs. 80%CHO and 60%CHO group (P<0.0005 and P=0.002, respectively). The 40%CHO group had also significantly higher ruminal pH than the 80%CHO (P=0.003). There were no other significant differences among the groups.

Health and performance outcomes

All the animals were healthy throughout the study. No animals were observed demonstrating clinical signs of any disease, including BRD. There were no differences in average daily gain among the groups (P values> 0.07). There were no differences in ruminal temperature as measured by ruminal boluses (P values > 0.8). The range of ruminal temperatures was from 34.7° C to 39° C.

Relative population changes of specific ruminal bacteria on days 7, 14 and 21

Day 7

Diet was a significant influencer of fold values for all bacterial species except *Streptococcus bovis* (P-values from <0.0005 to 0.018). Dunnett's T3 post-hoc was then performed for the remaining bacteria. For *Fibrobacter succinogenes*, while there was an apparent diet effect (P=0.011), there were no significant differences found between the diet comparisons. Similarly, there were no significant differences in fold change values for *Lactobacillus acidophilus* in post-hoc comparisons of diets, despite an apparently significant dietary effect (P=0.018).

Post-hoc comparison again found no difference between diets for *Megasphaera elsdenii* despite a highly significant diet effect (P<0.0005). The 80%CHO group had enormously higher fold change values than other groups (147-151 fold), producing numerous comparisons that approached significance: controls vs. 80%CHO (P=0.07), 40%CHO vs. 80%CHO (P=0.074), and 60%CHO vs.80%CHO (P=0.067). When a simple contrast was done to compare 80%CHO group to average of all others, the difference was large (mean of 149.3) and highly significant (P<0.0005).

A single post-hoc comparison was found to have significance for *Prevotella ruminocola*: control vs. 60%CHO group (P=0.032), with the 60%CHO group having higher mean fold change than

control (difference of 6.9). For *Ruminococcus flavefaciens*, a single significant difference was found on post-hoc; 80%CHO had lower mean fold change value than 60%CHO (P=0.022).

Day 14

For Day 14, fewer effects of diet were observed, with *Streptococcus bovis* again failing to be affected by diet (P=0.737), as was *Lactobacillus acidophilus* (P=0.166), and *Prevotella ruminocola* (P=0.055). On post-hoc analysis, *Fibrobacter succinogenes* had no significant differences among the groups. For *Lactobacillus acidophilus*, no comparisons had a P-value less than 0.1. For *Megasphaera elsdenii*, very large differences were observed between 80%CHO group and all other groups, this time achieving significance as individual comparisons. The 80%CHO group had a higher mean fold change value than all other groups (135-137 fold), (P=0.018-0.019). No other comparisons were significant. For *Prevotella ruminocola* and *Ruminococcus flavefaciens* no post-hoc analyses were significant (P>0.15).

Day 21

Diet had a significant impact on fold change values of all bacteria except *Prevotella ruminocola* (P=0.219) and *Streptococcus bovis* (P=0.095). For *Fibrobacter succinogenes*, no comparisons were significant. For *Lactobacillus acidophilus*, no comparisons were signicant. The fold change values for *Megasphaera elsdenii* seemed to produce the most significant comparisons. The 80%CHO group had significantly higher mean fold change values than the controls or the 40%CHO group (0.005 and 0.003, respectively). There was no difference in the fold change values between the 80%CHO group and the 60%CHO group (P=0.107). Comparisons among the other three diet combinations were not significant except for the 60%CHO having a notably higher mean fold change value than the 40%CHO group (mean fold change value difference of 9.2; P=0.045). For *Ruminococcus flavefaciens*, controls had significantly lower mean fold change

value than the 80%CHO group (P=0.017). Relative fold change values of ruminal bacteria are summarized in Figs.3-8.

Megasphaera elsdenii

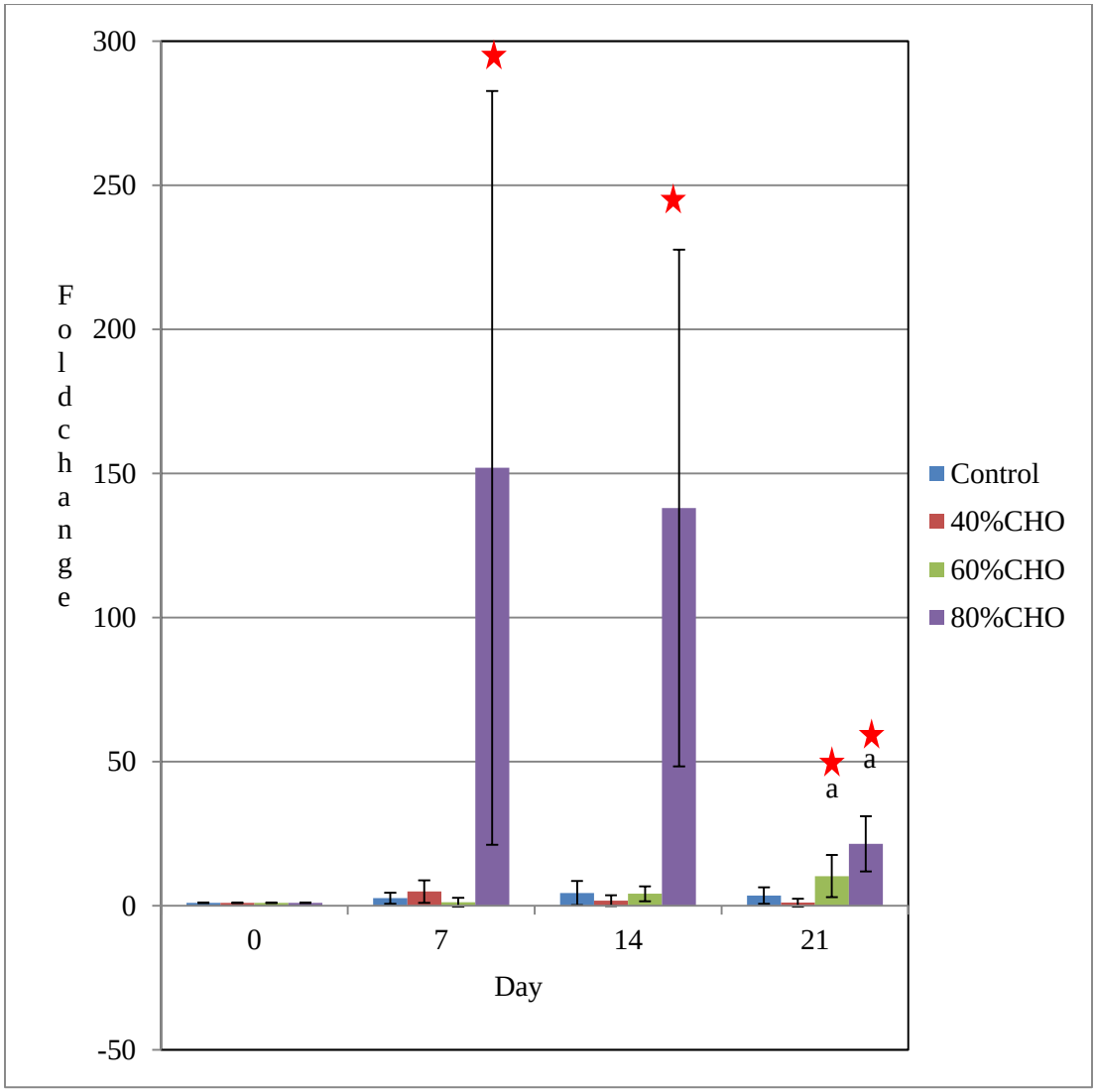

Fig.3. qRT-PCR- based relative population changes of *Megasphaera elsdenii* when varying amounts of fermentable carbohydrates were fed for 21 days. Relative change in population is shown as fold change. 16S was used for normalization and fold changes in bacterial populations were calculated as described in materials and methods. Comparisons were made among the groups within the day of sampling. An asterisk indicates a group with a mean fold change value that is significantly different from the control group. Groups with the same letter are not different from each other. The data represent the geometric of 8 animals/group.

Prevotella ruminocola

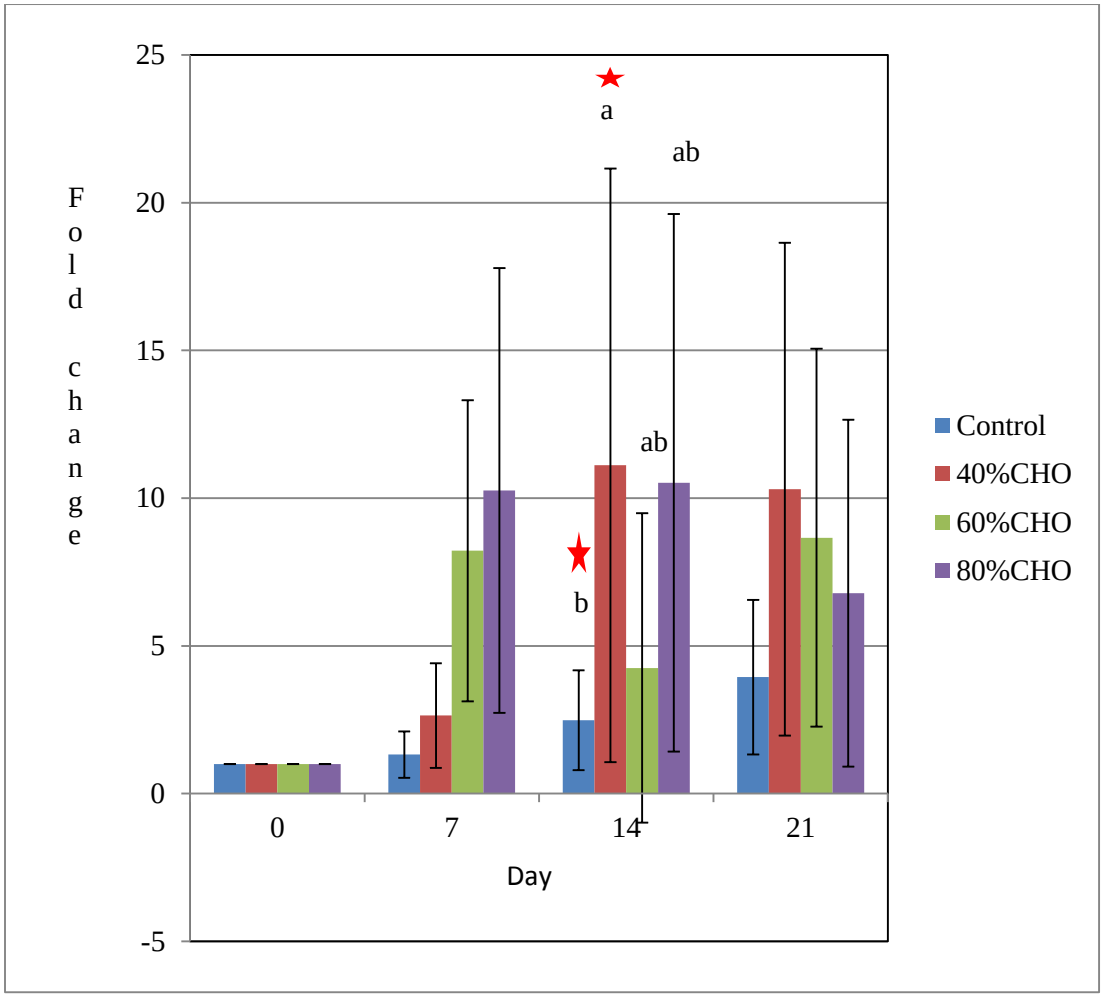

Fig.4. qRT-PCR- based relative population changes of *Prevotella ruminocola* when varying amounts of fermentable carbohydrates were fed for 21 days. Relative change in population is shown as fold change. 16S was used for normalization and fold changes in bacterial populations were calculated as described in materials and methods. Comparisons were made among the groups within the day of sampling. An asterisk indicates a group with a mean fold change value that is significantly different from the control group. Groups with the same letter are not different from each other. The data represent the geometric of 8 animals/group.

Ruminococcus flavefaciens

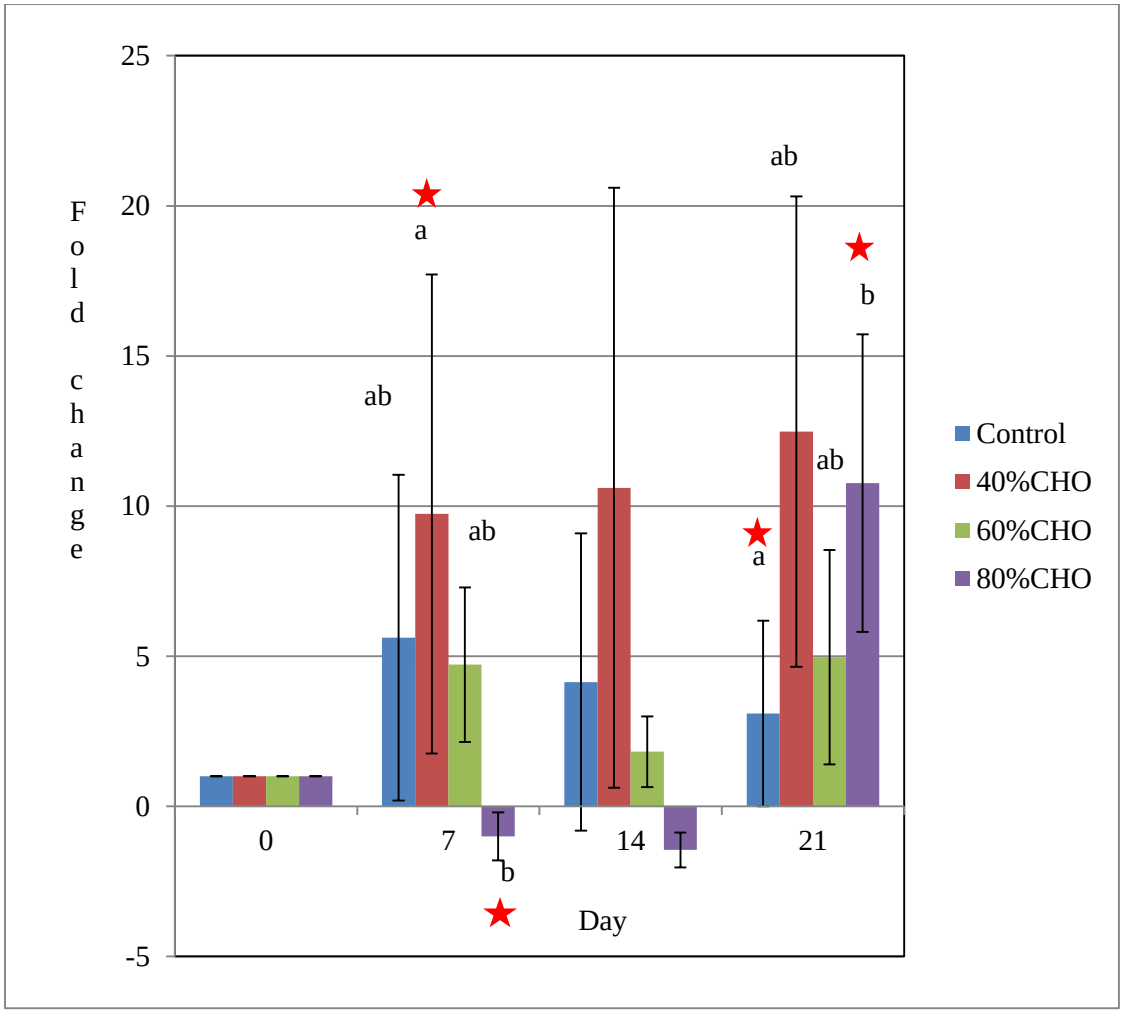

Fig.5. qRT-PCR- based relative population changes of *Ruminococcus flavefaciens* when varying amounts of fermentable carbohydrates were fed for 21 days. Relative change in population is shown as fold change. 16S was used for normalization and fold changes in bacterial populations were calculated as described in materials and methods. Comparisons were made among the groups within the day of sampling. An asterisk indicates a group with a mean fold change value that is significantly different from the control group. Groups with the same letter are not different from each other. The data represent the geometric of 8 animals/group.

Streptococcus bovis

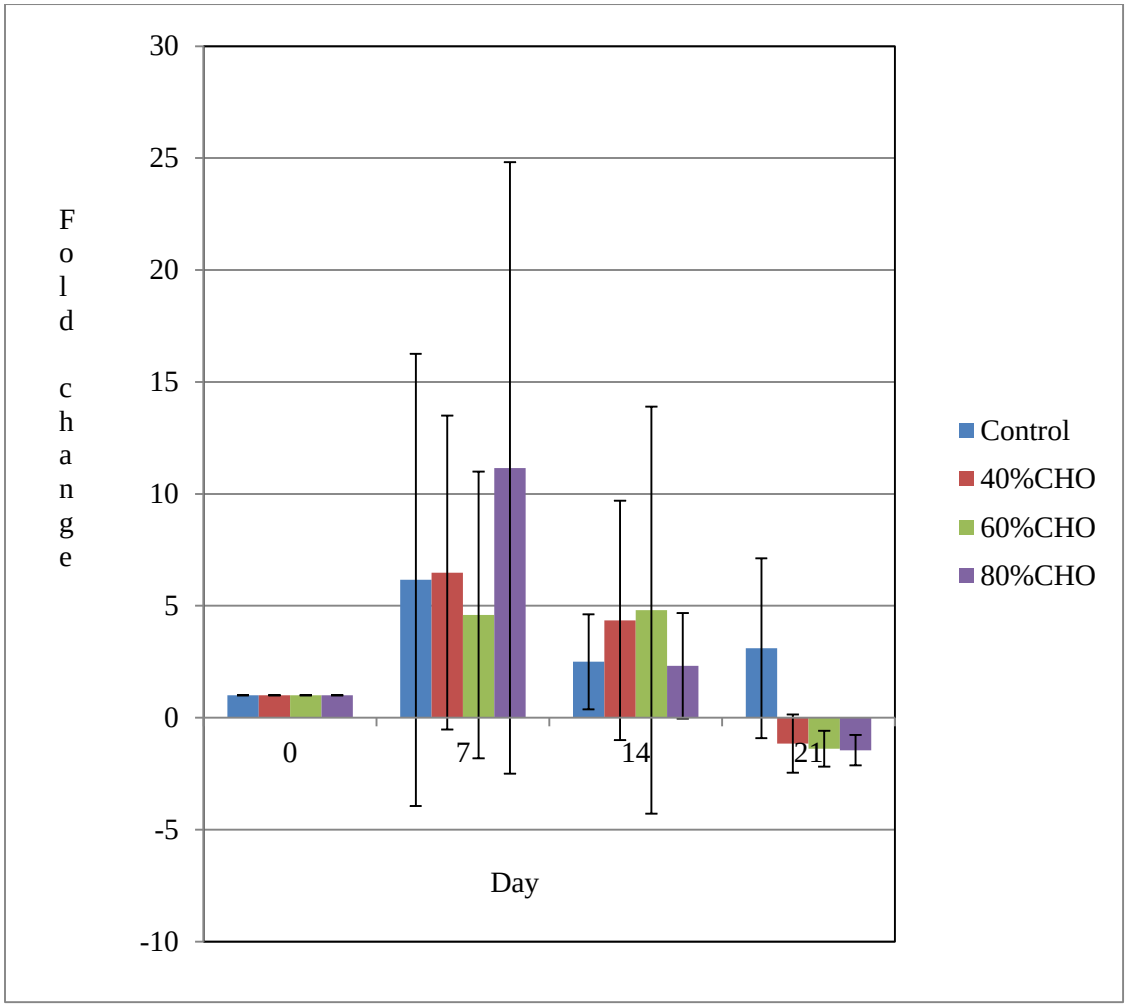

Fig.6. qRT-PCR- based relative population changes of *Streptococcus bovis* when varying amounts of fermentable carbohydrates were fed for 21 days. Relative change in population is shown as fold change. 16S was used for normalization and fold changes in bacterial populations were calculated as described in materials and methods. Comparisons were made among the groups within the day of sampling. An asterisk indicates a group with a mean fold change value that is significantly different from the control group. Groups with the same letter are not different from each other. The data represent the geometric of 8 animals/group.

Lactobacillus acidophilus

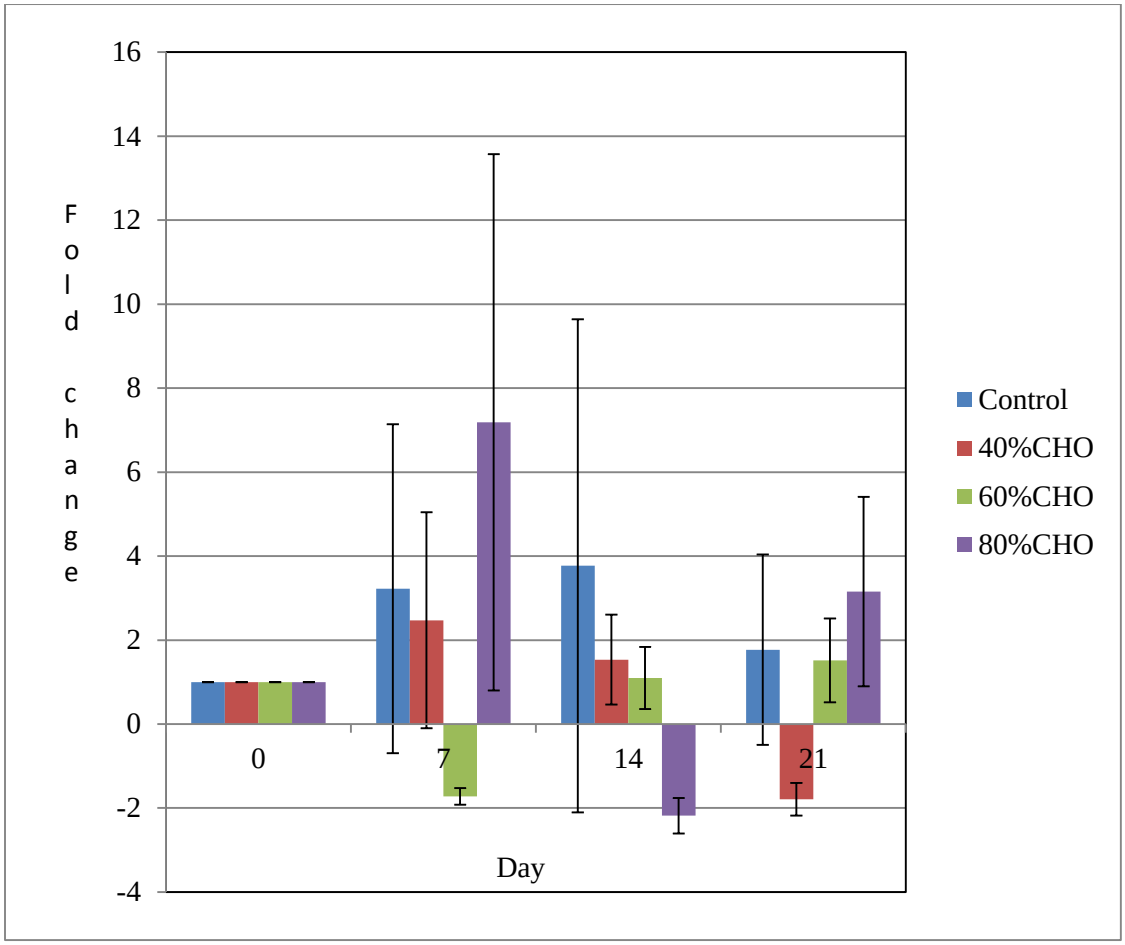

Fig.7. qRT-PCR- based relative population changes of *Lactobacillus acidophilus* when varying amounts of fermentable carbohydrates were fed for 21 days. Relative change in population is shown as fold change. 16S was used for normalization and fold changes in bacterial populations were calculated as described in materials and methods. Comparisons were made among the groups within the day of sampling. An asterisk indicates a group with a mean fold change value that is significantly different from the control group. Groups with the same letter are not different from each other. The data represent the geometric of 8 animals/group.

Fibrobacter Succinogenes

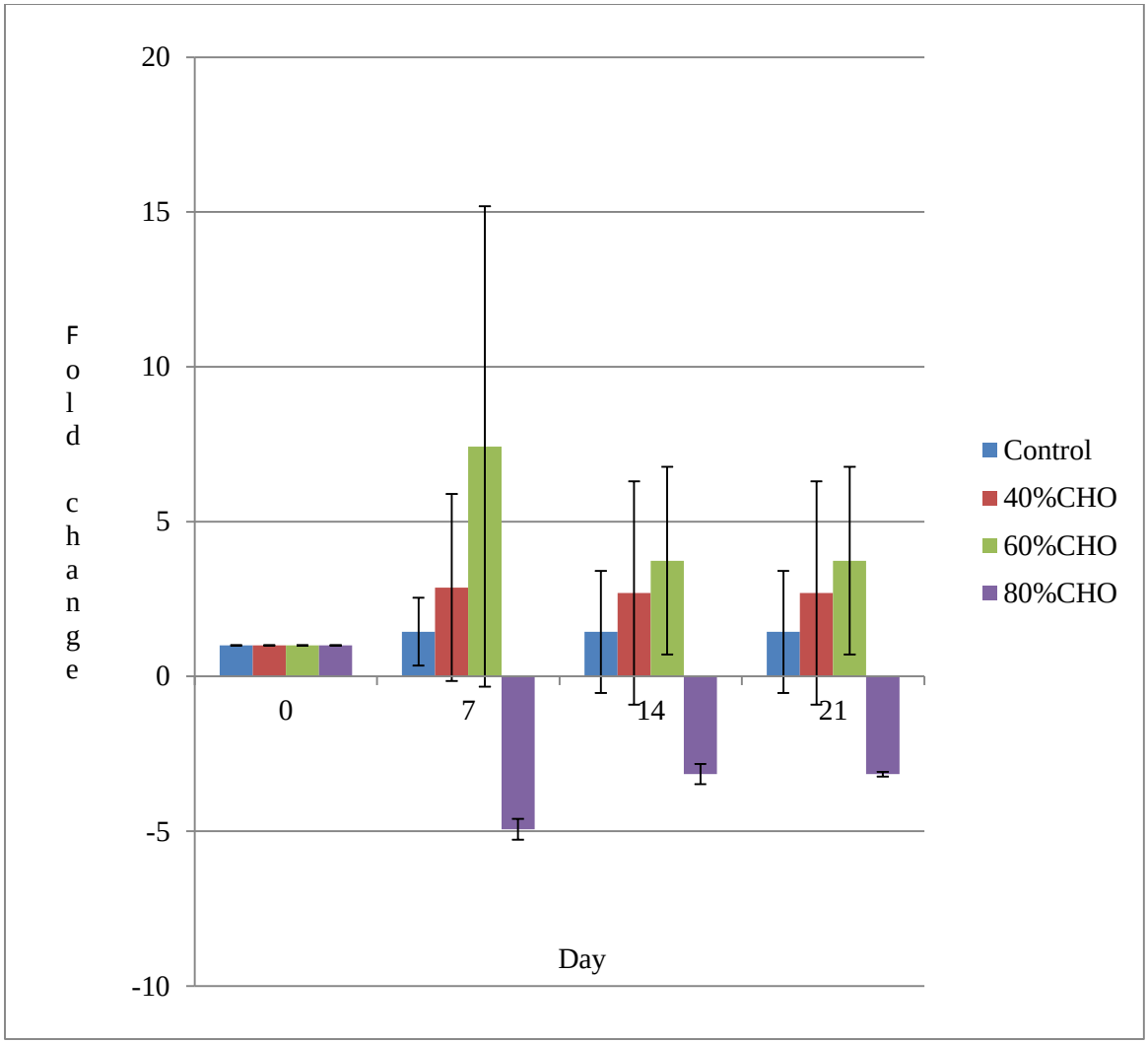

Fig.8. qRT-PCR- based relative population changes of *Fibrobacter succinogenes* when varying amounts of fermentable carbohydrates were fed for 21 days. Relative change in population is shown as fold change. 16S was used for normalization and fold changes in bacterial populations were calculated as described in materials and methods. Comparisons were made among the groups within the day of sampling. An asterisk indicates a group with a mean fold change value that is significantly different from the control group. Groups with the same letter are not different from each other. The data represent the geometric of 8 animals/group.

Relative cytokine mRNA expression in BALF

Day 7

Diet had a significant effect ($P<0.0005$) on all cytokine fold change values except for TGFβ1 ($P=0.187$). Post-hoc analysis found IL-1β fold change values to be significantly lower in the control group and the 40%CHO group than in 80%CHO or 60%CHO group ($P<0.0005$). There was no statistically significant difference between the controls and the 40%CHO group ($P=0.935$). The mean fold change values between the 60%CHO group and the 80%CHO were not significantly different from each other ($P=0.963$). For IL-8, post-hoc analysis found the fold change values to be significantly different among all four groups ($P<0.0005$ to $P=0.027$).

For IL-10, controls had lower mean fold change value than the 80%CHO group ($P=0.004$). There was no difference between control and the 40%CHO group. The mean IL-10 fold change value for the 40%CHO was significantly different from that of the 80%CHO group ($P=0.003$). For IL-10, the mean fold change value for the 60%CHO group was not significantly different from any other groups ($P= 0.087$).

For TNF-α, controls and the 40%CHO group had significantly lower fold change values than the 60%CHO and the 80%CHO groups ($P=0.001$ and 0.049 respectively). There was no difference in fold change values between controls and the 40%CHO group ($P=0.228$). There was no difference between the 80%CHO group and the 60%CHO group.

Day 14

All cytokines except TGFβ1 again showed a significant relationship with diet ($P<0.005$ to $P=0.01$). IL-1β showed a pattern very similar to day 7. Both the control group and the 40%CHO group had fold change values significantly lower than the 60%CHO and the 80%CHO groups

(P<0.0005 in all cases). The control group was not statistically different from the 40%CHO group (P=0.999). There was no difference between the 60%CHO and the 80%CHO group (P=0.952).

For IL-8, the mean fold changes for the controls and the 40%CHO group were both lower than the mean fold change for the 80%CHO group (P=0.02 and P=0.014,respectively). There were no other significant differences. For IL-10, the fold changes for the controls and the 40%CHO group were lower than the mean fold change for the 80%CHO group (P=0.037 and P=0.07, respectively), but not different from each other or the 60%CHO group (P=0.189 and P=0.925, respectively). For TNF-α, the mean fold change value for the controls was less than the mean fold change value for the 60%CHO or 40%CHO group (P=0.001 and P<0.0005, respectively). The 40%CHO group had lower mean fold change value than the 80%CHO group but not different from the controls or the 60%CHO group (P=0.065). The 80%CHO group had significantly higher mean fold change value than any other group (P<0.0005).

Day 21

Similar to days 7 and 14, all cytokine fold change values were significantly associated with diet group except TGFβ1. For the others, P-values ranged from <0.0005 to 0.024. There was a notable change in the fold change value comparisons for IL-1β. The order for day 21 was now basically reversed from days 7 and 14. The control group had slightly higher mean fold change value than the 60%CHO and the 80%CHO groups (P=0.009 and P= 0.004, respectively). Similarly, the fold change value of the 40%CHO group was modestly higher than for the 80%CHO group (P=0.032). Unlike for earlier days, the fold change for the 40%CHO group was not significantly different from that of the 60%CHO group (P=0.052). There was no difference between the control group and the 40%CHO (P=0.496). There was no difference between the 40%CHO group and the 60%CHO group (P= 0.052), or 60%CHO and 80%CHO group (P=0.986).

For IL-8, the mean fold change for the controls remained numerically lower compared to other diet groups, but was only significantly different from the 60%CHO group (P=0.012) There were no other significant differences found in post-hoc analysis. For IL-10, the only significant difference was between the 40% CHO and the 80%CHO group, with the 40%CHO group having a lower fold change value (P=0.035).

For TNF-α, no significant associations were present. Results of the fold change values of cytokines in BALF are summarized in Figs.9-13.

BALF IL-1β

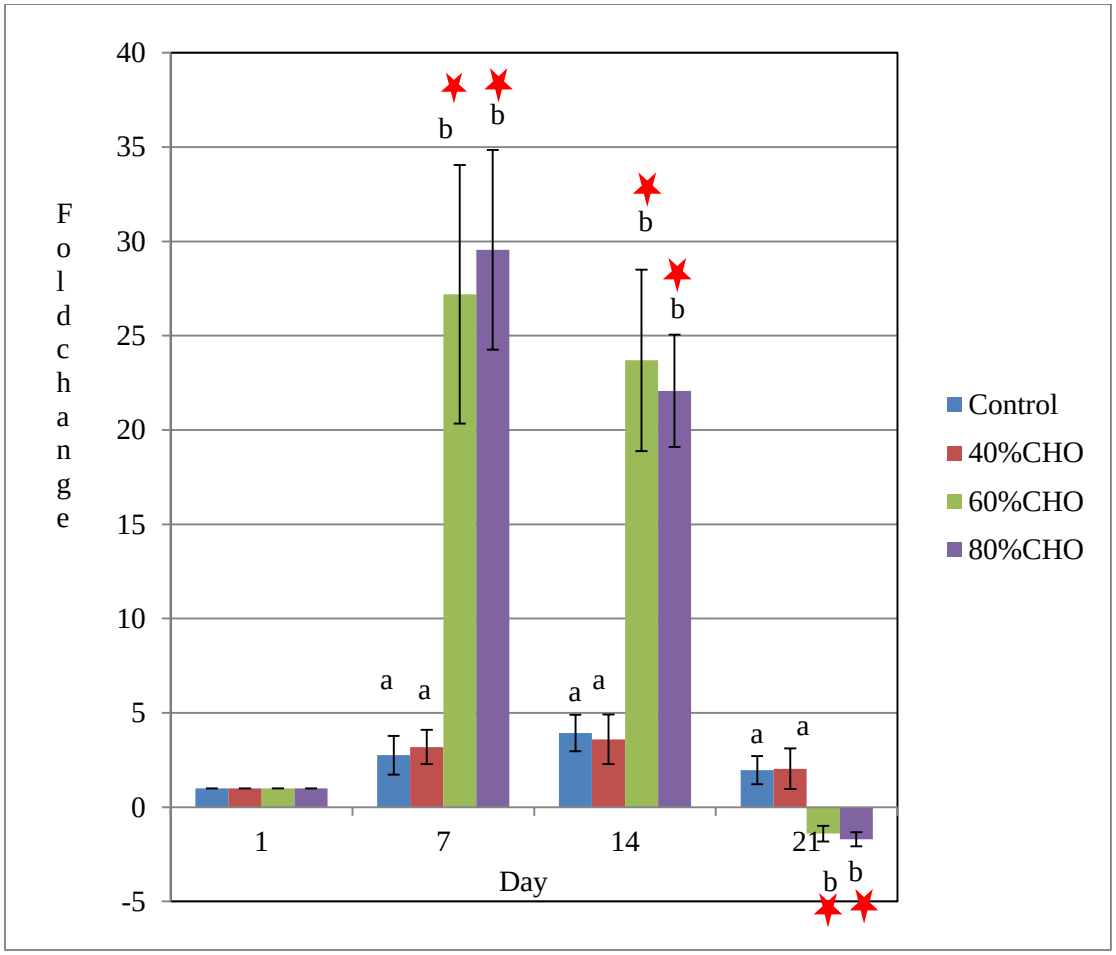

Fig.9. qRT-PCR- based relative expression of IL-1β in BALF when varying amounts of fermentable carbohydrates were fed for 21 days. Relative expression is shown as fold change. Bovine actin was used for normalization and fold changes of cytokine expression were calculated as described in materials and methods. Comparisons were made among the groups within the day of sampling. An asterisk indicates a group with a mean fold change value that is significantly different from the control group. Groups with the same letter are not different from each other. The data represent the geometric of 8 animals/group.

BALF TNF

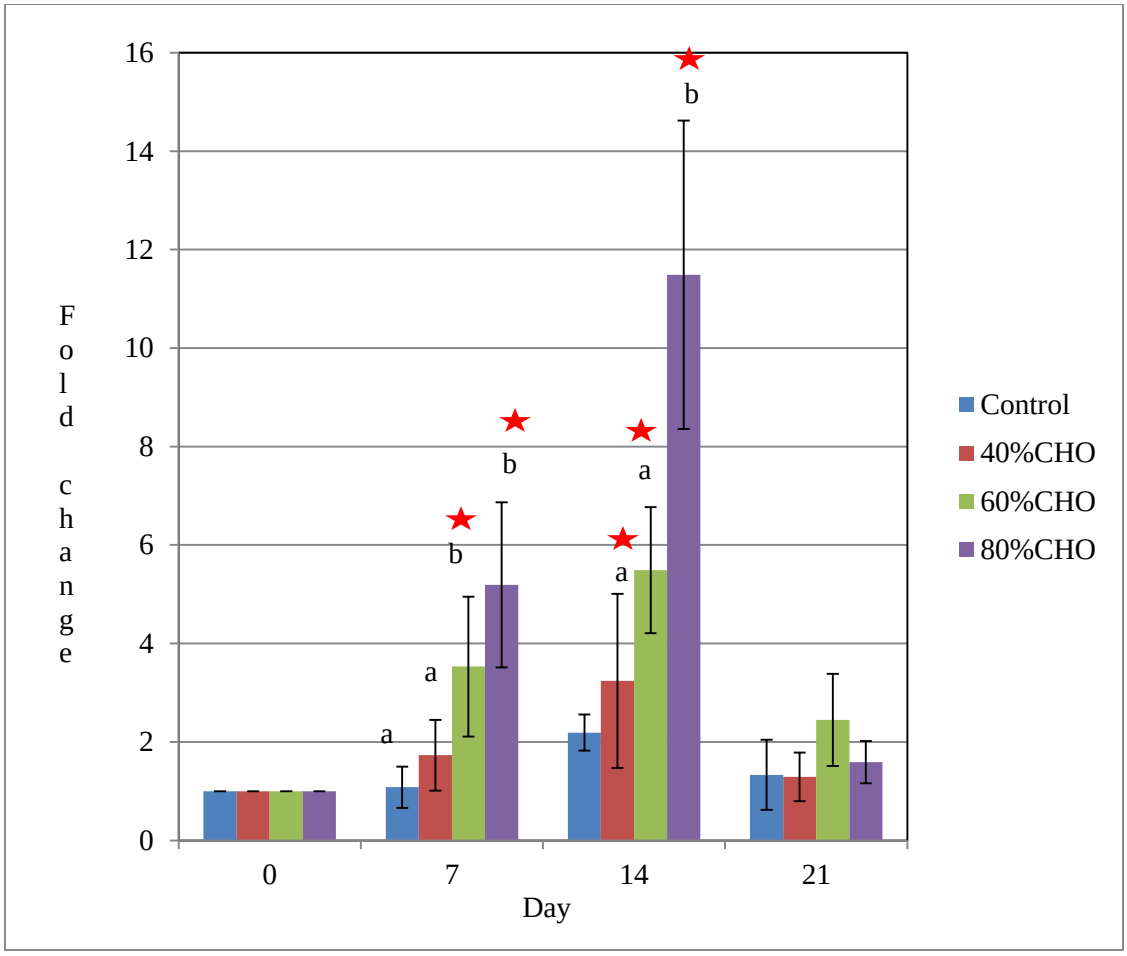

Fig.10. qRT-PCR- based relative expression of TNF in BALF when varying amounts of fermentable carbohydrates were fed for 21 days. Relative expression is shown as fold change. Bovine actin was used for normalization and fold changes of cytokine expression were calculated as described in materials and methods. Comparisons were made among the groups within the day of sampling. An asterisk indicates a group with a mean fold change value that is significantly different from the control group. Groups with the same letter are not different from each other. Groups with the same letter are not different from each other. The data represent the geometric of 8 animals/group.

BALF IL-8

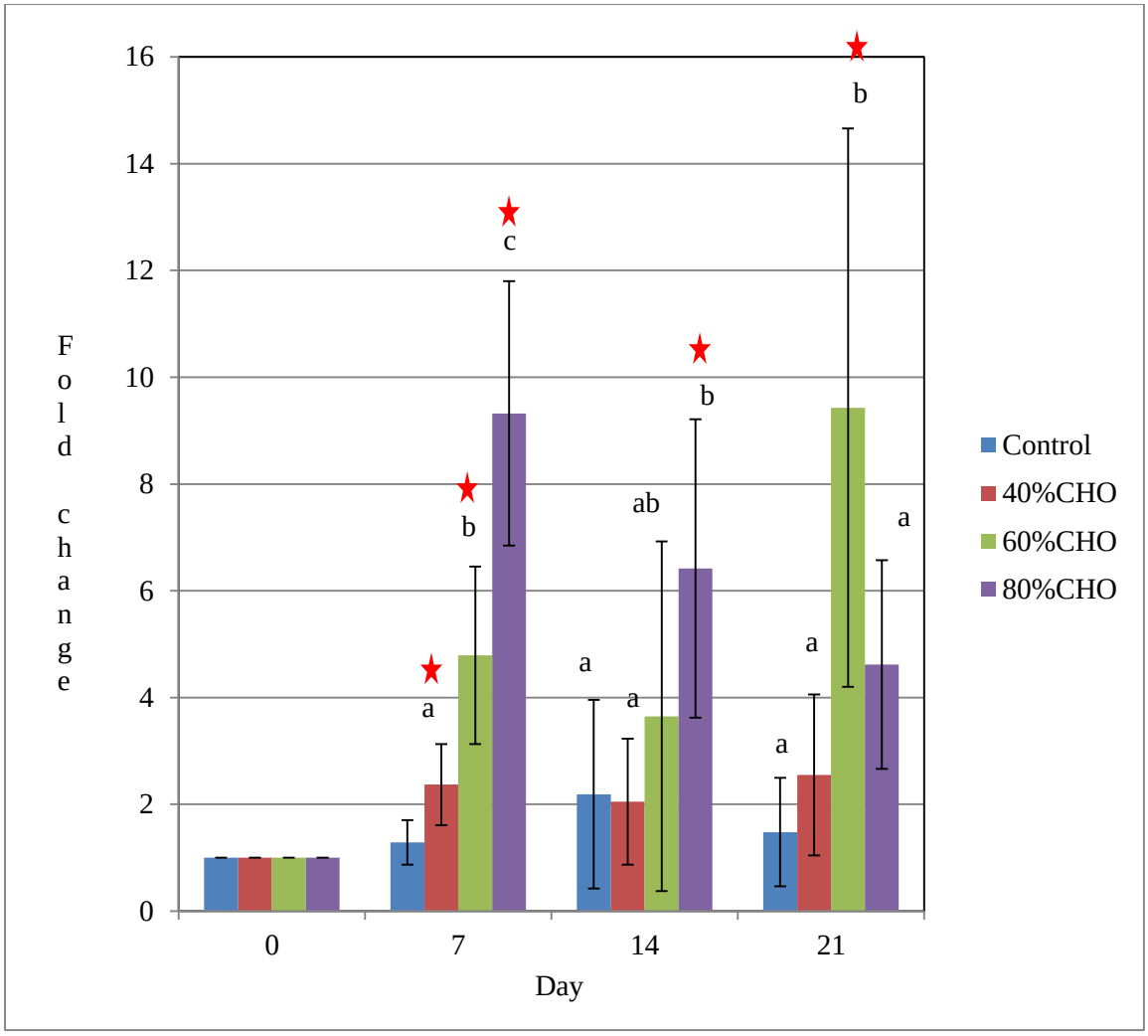

Fig.11. qRT-PCR- based relative expression of IL-8 in BALF when varying amounts of fermentable carbohydrates were fed for 21 days. Relative expression is shown as fold change. Bovine actin was used for normalization and fold changes of cytokine expression were calculated as described in materials and methods. Comparisons were made among the groups within the day of sampling. An asterisk indicates a group with a mean fold change value that is significantly different from the control group. Groups with the same letter are not different from each other. The data represent the geometric of 8 animals/group.

BALF IL-10

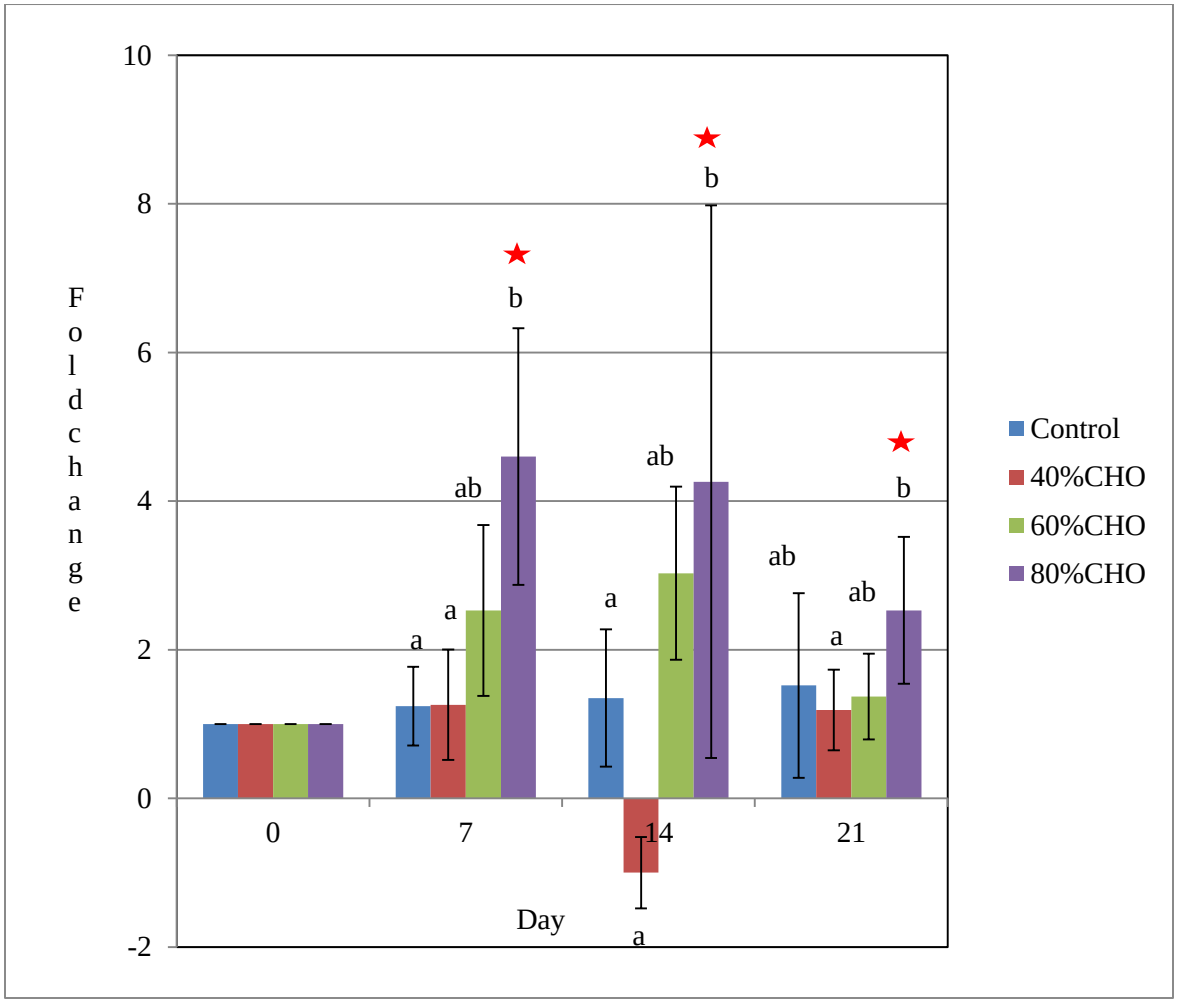

Fig.12. qRT-PCR- based relative expression of IL-10 in BALF when varying amounts of fermentable carbohydrates were fed for 21 days. Relative expression is shown as fold change. Bovine actin was used for normalization and fold changes of cytokine expression were calculated as described in materials and methods. Comparisons were made among the groups within the day of sampling. An asterisk indicates a group with a mean fold change value that is significantly different from the control group. Groups with the same letter are not different from each other. The data represent the geometric of 8 animals/group.

BALF TGFβ1

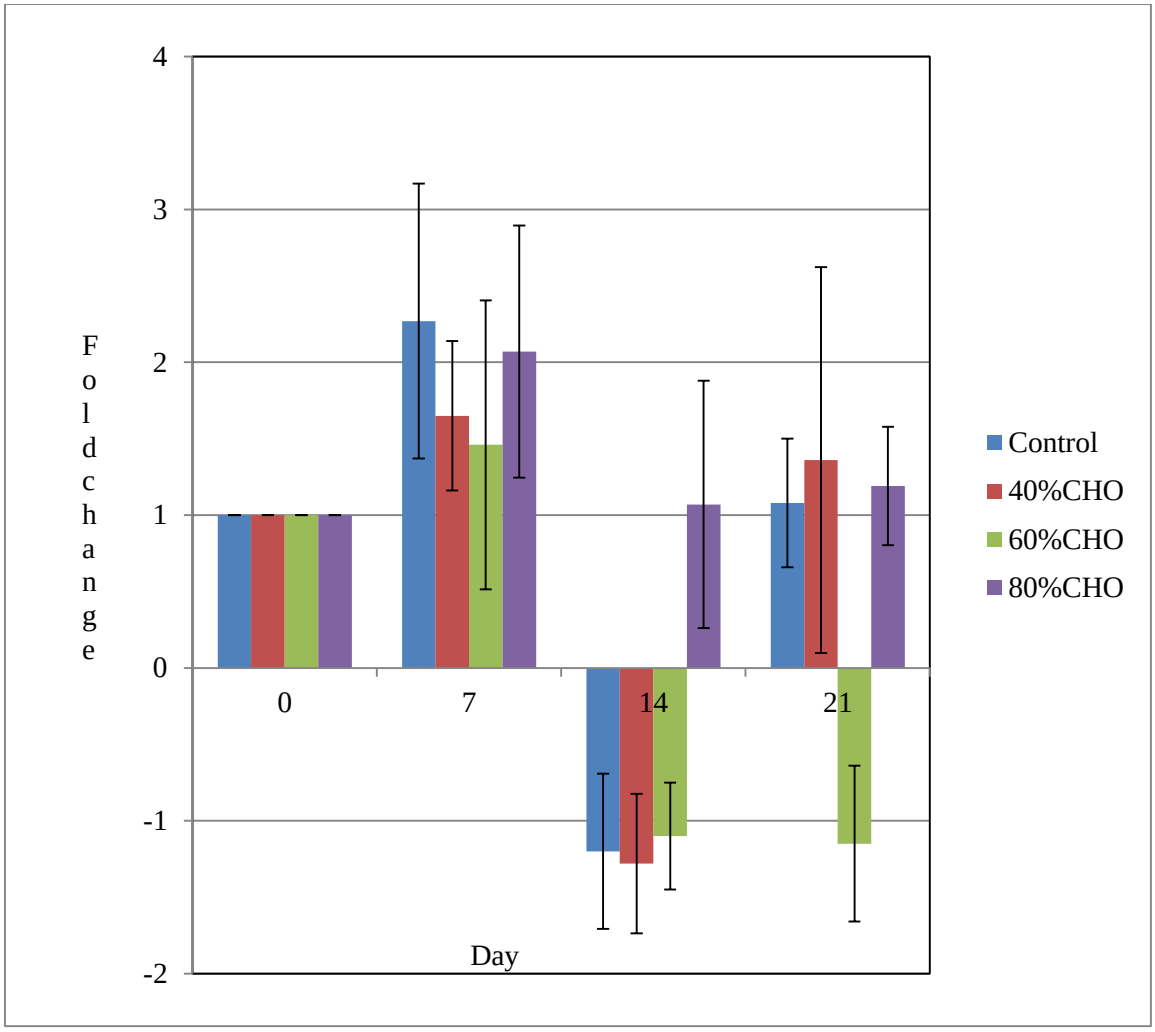

Fig.13. qRT-PCR- based relative expression of TGFβ1 in BALF when varying amounts of fermentable carbohydrates were fed for 21 days. Relative expression is shown as fold change. Bovine actin was used for normalization and fold changes of cytokine expression were calculated as described in materials and methods. Comparisons were made among the groups within the day of sampling. An asterisk indicates a group with a mean fold change value that is significantly different from the control group. Groups with the same letter are not different from each other. The data represent the geometric of 8 animals/group.

Relative cytokine mRNA expression in blood

Day 7

Diet was significantly associated with blood cytokine fold change values for all cytokines except IL-10 (P=0.119). Post hoc analysis for IL-1β found a notably lower fold change value for controls compared to the 60%CHO group and the 80%CHO group (P<0.0005 for all comparisons). There was no difference between the controls and the 40%CHO group (P=0.935), nor between the 60%CHO group and the 80%CHO group (P=0.963). For IL-8, there were no significant differences among the groups (All P- values > 0.20). For TGFβ1 no significant differences were observed on post-hoc analysis. For TNF-α, the control group had significantly lower fold change values than both 60%CHO and the 80%CHO (P=0.026 and P=0.01, respectively).

Day 14

There were significant associations between diet and cytokine fold change values for all cytokines except IL-8 (P=0.243) and TGFβ1 (P=0.573). Post-hoc analysis of IL-1β found that the mean fold change values for the control group and the 40%CHO group were significantly lower than for the 60%CHO and the 80%CHO group (P<0.0005 for all comparisons). There were no significant difference between the controls and the 40%CHO fold changes (P=0.999), nor between the 80%CHO group and the 60%CHO group (P=0.952). For IL-10, the mean fold change value for the control group was significantly lower than for the 40%CHO group (P=0.005). There were no other significant associations among the groups (all p-values >0.40). For TNF-α, controls had lower mean fold change value than all other groups (P=0.004, P=0.001, and P=0.002 for 40%CHO, 60%CHO and 80%CHO group, respectively). The 40%CHO group had lower mean fold change value than the 60%CHO group (P=0.006), but not different from the 80%CHO group (P=0.083).

Day 21

Significant association between diet and cytokine fold change values were observed for IL-1β (<0.0005) and TNF-α (0.028), but for no other cytokines (all >0.25). For IL-1β, the mean fold change in the control group and the 40%CHO group were both higher than in the 80%CHO group (P=0.004 and P=0.032, respectively). The mean fold change value for the control group was also higher than for the 60%CHO group (P=0.009). For TNF-α, no significant differences were found in post-hoc analysis. . Results of the fold change values of cytokines in blood are summarized in Figs.14-18.

Blood IL-1β

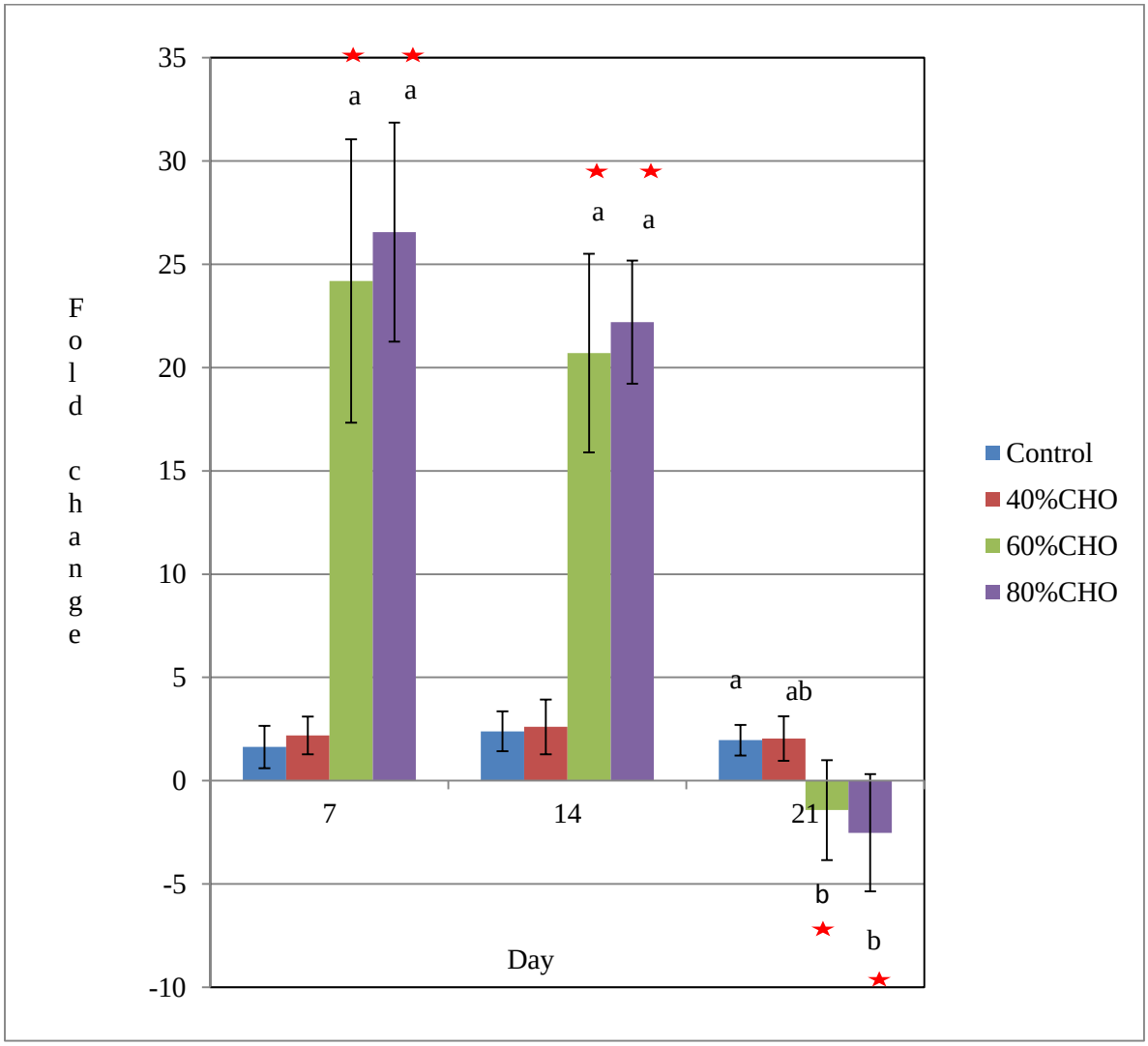

Fig.14. qRT-PCR- based relative expression of IL-1β in blood when varying amounts of fermentable carbohydrates were fed for 21 days. Relative expression is shown as fold change. Bovine actin was used for normalization and fold changes of cytokine expression were calculated as described in materials and methods. Comparisons were made among the groups within the day of sampling. An asterisk indicates a group with a mean fold change value that is significantly different from the control group. Groups with the same letter are not different from each other. The data represent the geometric of 8 animals/group.

Blood TNF

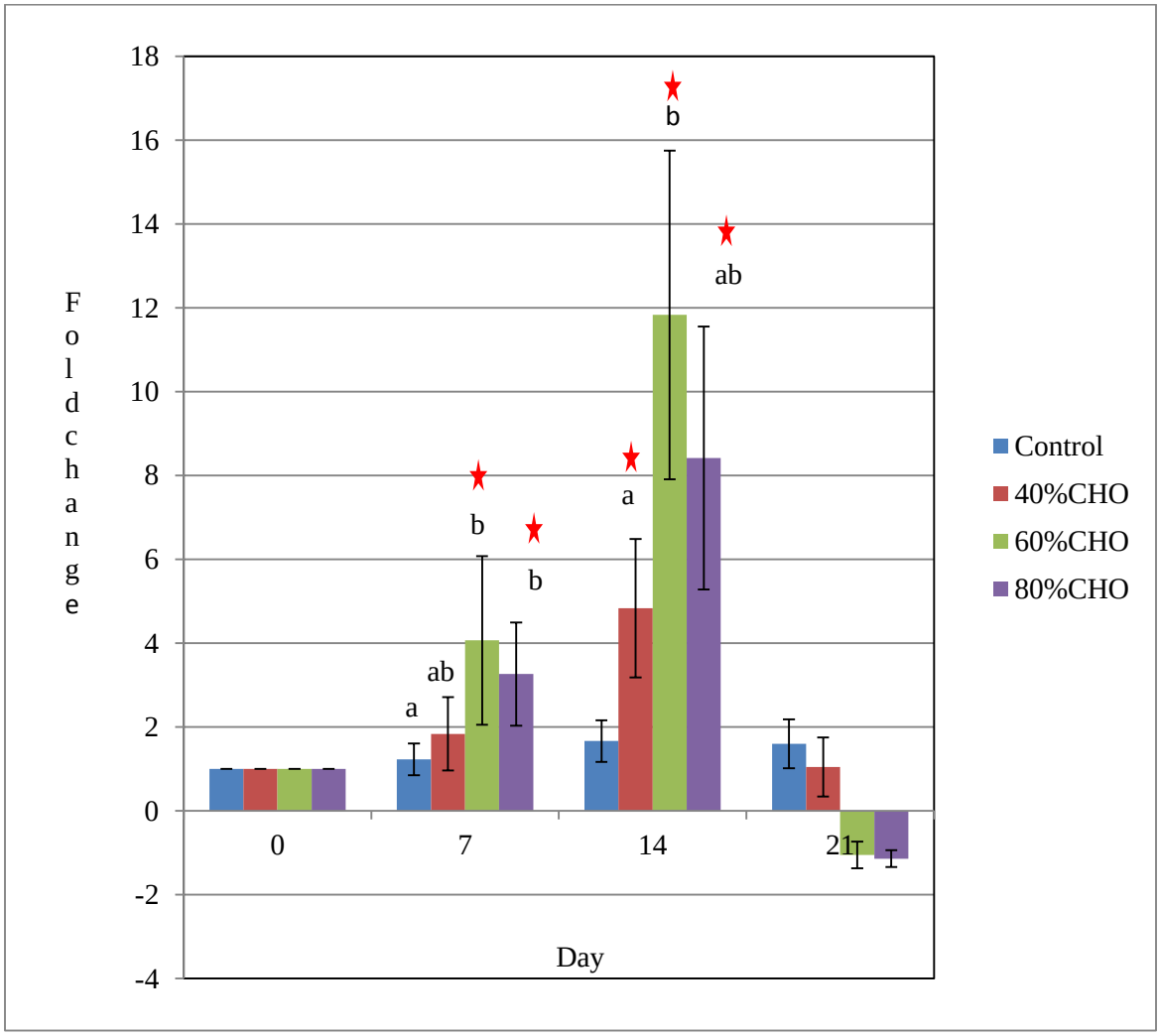

Fig.15. qRT-PCR- based relative expression of TNF in blood when varying amounts of fermentable carbohydrates were fed for 21 days. Relative expression is shown as fold change. Bovine actin was used for normalization and fold changes of cytokine expression were calculated as described in materials and methods. Comparisons were made among the groups within the day of sampling. An asterisk indicates a group with a mean fold change value that is significantly different from the control group. Groups with the same letter are not different from each other. The data represent the geometric of 8 animals/group.

Blood IL-8

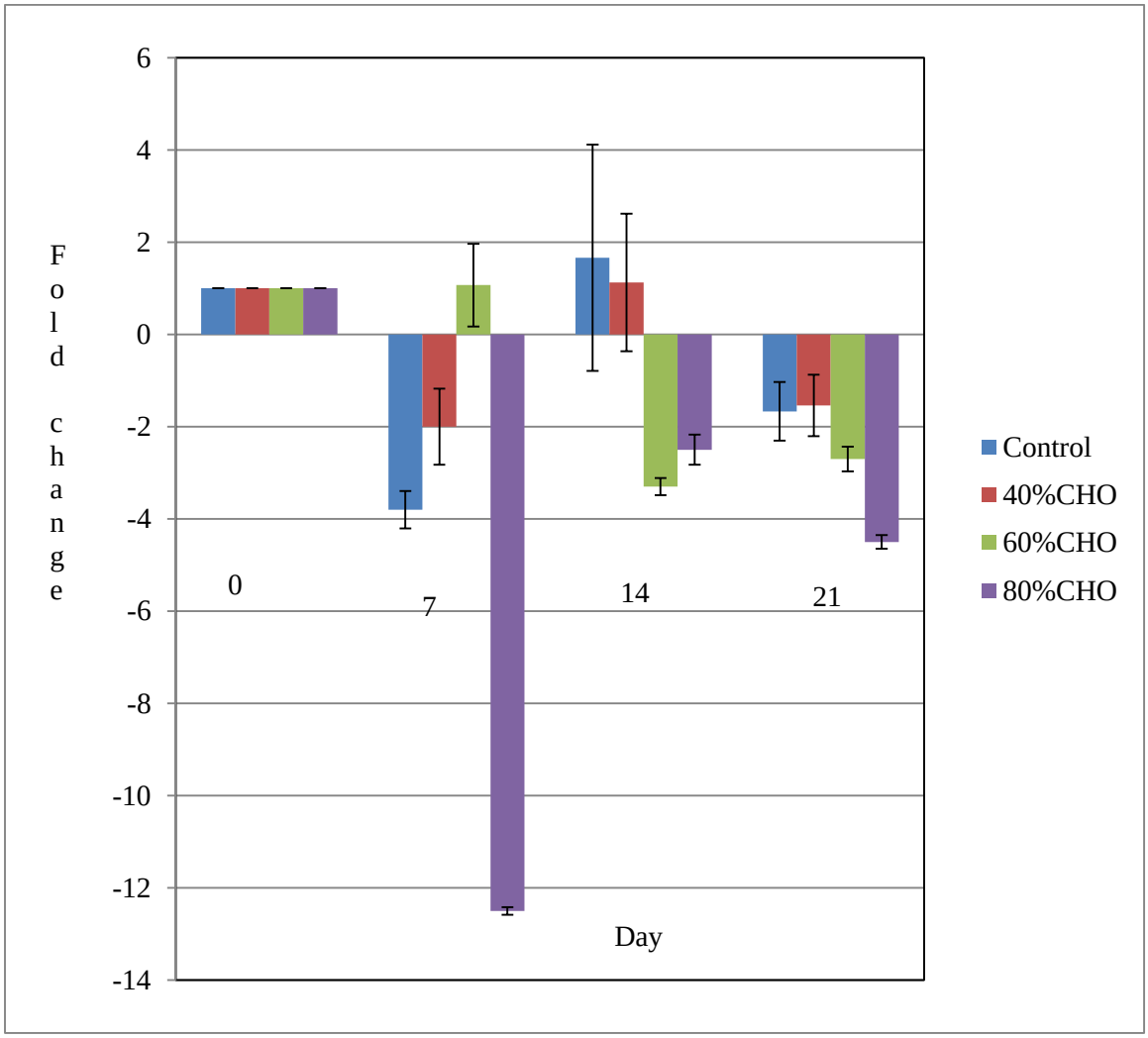

Fig.16. qRT-PCR- based relative expression of IL-8 in blood when varying amounts of fermentable carbohydrates were fed for 21 days. Relative expression is shown as fold change. Bovine actin was used for normalization and fold changes of cytokine expression were calculated as described in materials and methods. Comparisons were made among the groups within the day of sampling. An asterisk indicates a group with a mean fold change value that is significantly different from the control group. Groups with the same letter are not different from each other. The data represent the geometric of 8 animals/group.

Blood IL-10

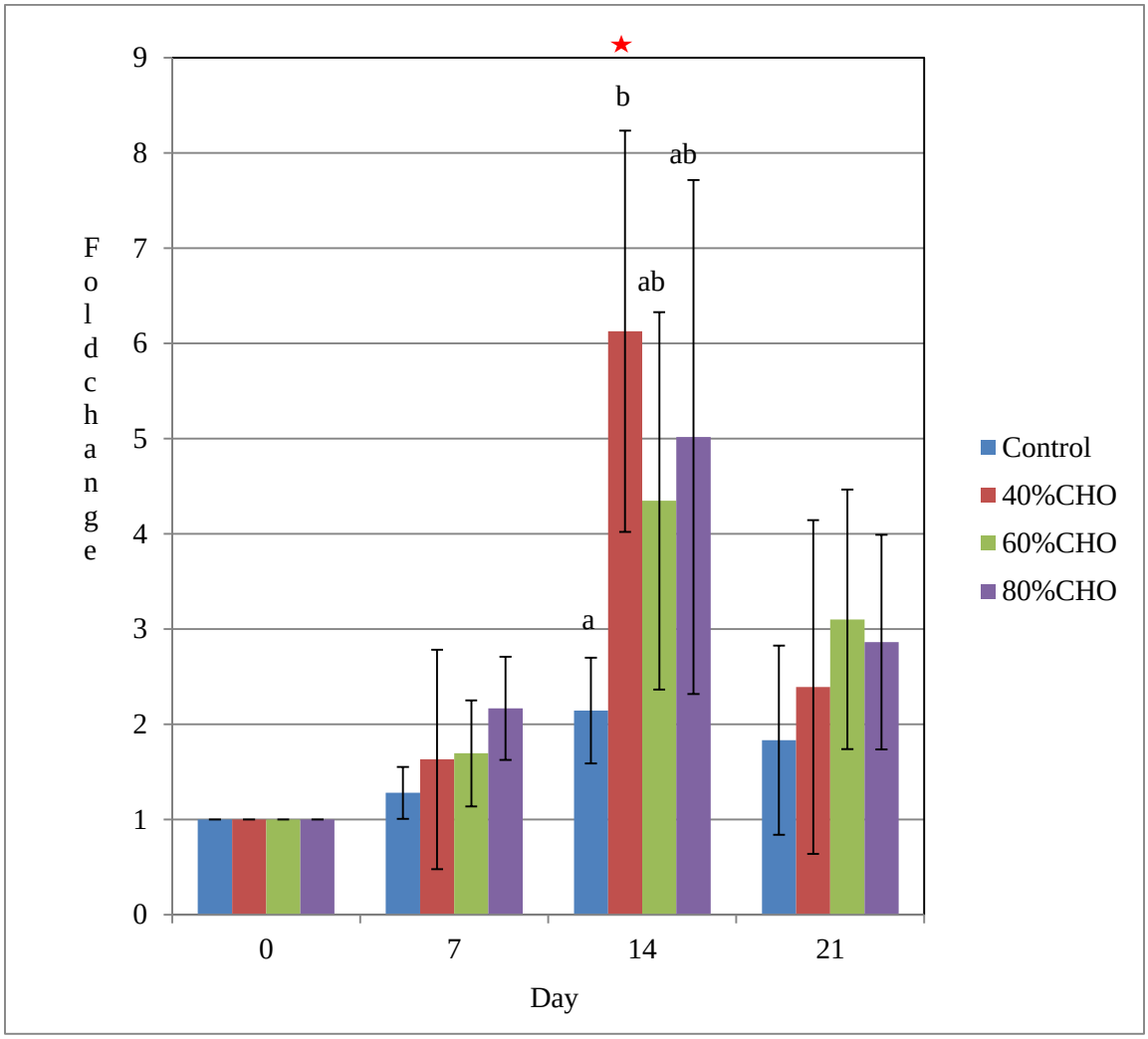

Fig.17. qRT-PCR- based relative expression of IL-10 in blood when varying amounts of fermentable carbohydrates were fed for 21 days. Relative expression is shown as fold change. Bovine actin was used for normalization and fold changes of cytokine expression were calculated as described in materials and methods. Comparisons were made among the groups within the day of sampling. An asterisk indicates a group with a mean fold change value that is significantly different from the control group. Groups with the same letter are not different from each other. The data represent the geometric of 8 animals/group.

Blood TGFβ1

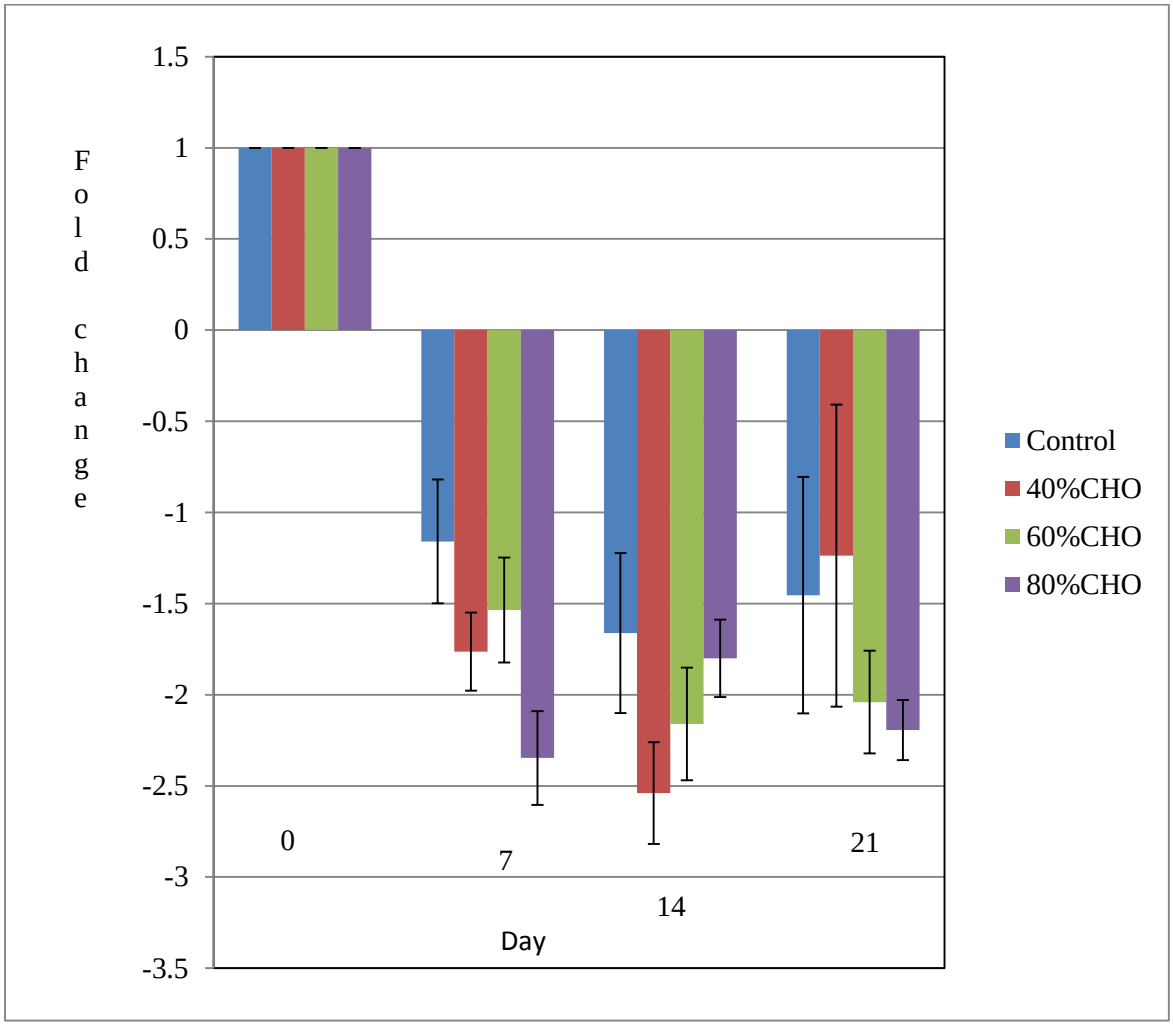

Fig.18. qRT-PCR- based relative expression of TGFβ1 in blood when varying amounts of fermentable carbohydrates were fed for 21 days. Relative expression is shown as fold change. Bovine actin was used for normalization and fold changes of cytokine expression were calculated as described in materials and methods. Comparisons were made among the groups within the day of sampling. An asterisk indicates a group with a mean fold change value that is significantly different from the control group. Groups with the same letter are not different from each other. The data represent the geometric of 8 animals/group.

Relationship between BALF and blood cytokine mRNA expression

Day 7

There was a positive correlation between IL-1β mRNA expression in blood and BALF (r=1, P<0.0005). Similarly, expression of TNF in blood was positively correlated with its expression in BALF (r=0.358, P=0.044). IL-8 and IL-10 mRNA expression in blood was not correlated with BALF IL-8 and BALF IL-10 mRNA expression (r=0.016, P=0.93; r= 0.309, P=0.086, respectively). Similarly to IL-8 and IL-10, the expression of TGFβ1 was not correlated with its expression in BALF(r=0.206, P=0.258).

Day 14

Similar to day 7, there was a positive correlation between IL-1β mRNA expression in blood BALF (r=1, P<0.0005). The expression of TNF mRNA in blood was also correlated with its expression in BALF (r= 0.399, P= 0.024).There was no correlation in expression of IL-8, IL-10 and TGFβ1 between blood and BALF (r=-0.141, P=0.441; r= 0.152, P=405; r= -0.039, P=0.833; respectively).

Day 21

The pattern of cytokine mRNA expression in blood and BALF for day 21 was similar to days 7 and 14 except for TNF. There was a positive correlation between IL-1β mRNA expression in blood and BALF (r=1, P<0.0005). Unlike on days 7 and 14, the expression of TNF-α mRNA in blood was not correlated with its expression in BALF (r= -0.137, P= 0.455). There was no correlation in expression of IL-8, IL-10 and TGFβ between blood and BALF (r=-0.140, P=0.444; r= 0.029, P=0.875; r= -0.223, P=0.220; respectively).

Ruminal fluid endotoxin concentration

The method was validated and met all the preset conditions as described in the methods section. Fig.17 is an example of a standard curve generated when endotoxin was quantified in ruminal fluid using. The mean endotoxin concentration in the ruminal fluid was depended on diet and time ($P=0.0019$ and $P<0.0001$, respectively).There was a significant interaction between diet and time ($P=<0.0001$). The four groups had similar ruminal fluid endotoxin levels on days 0 and 7 ($P=0.992$ and $P=0.146$, respectively). The mean ruminal fluid endotoxin concentration was different among the groups on days 14 and 21($P=0.0022$ and $P<0.0001$, respectively). The control group diet did not have a significant effect on the concentration of ruminal fluid concentration ($P=0.687$). The 40%CHO, 60%CHO and 80%CHO group diets significantly increased the concentration of endotoxin in the ruminal fluid ($P=0.0092$, $P<0.0001$, and $P<0.0001$, respectively). Figs 20 and 21 show the means of endotoxin concentration over time 21 days in ruminal fluid and plasma respectively.

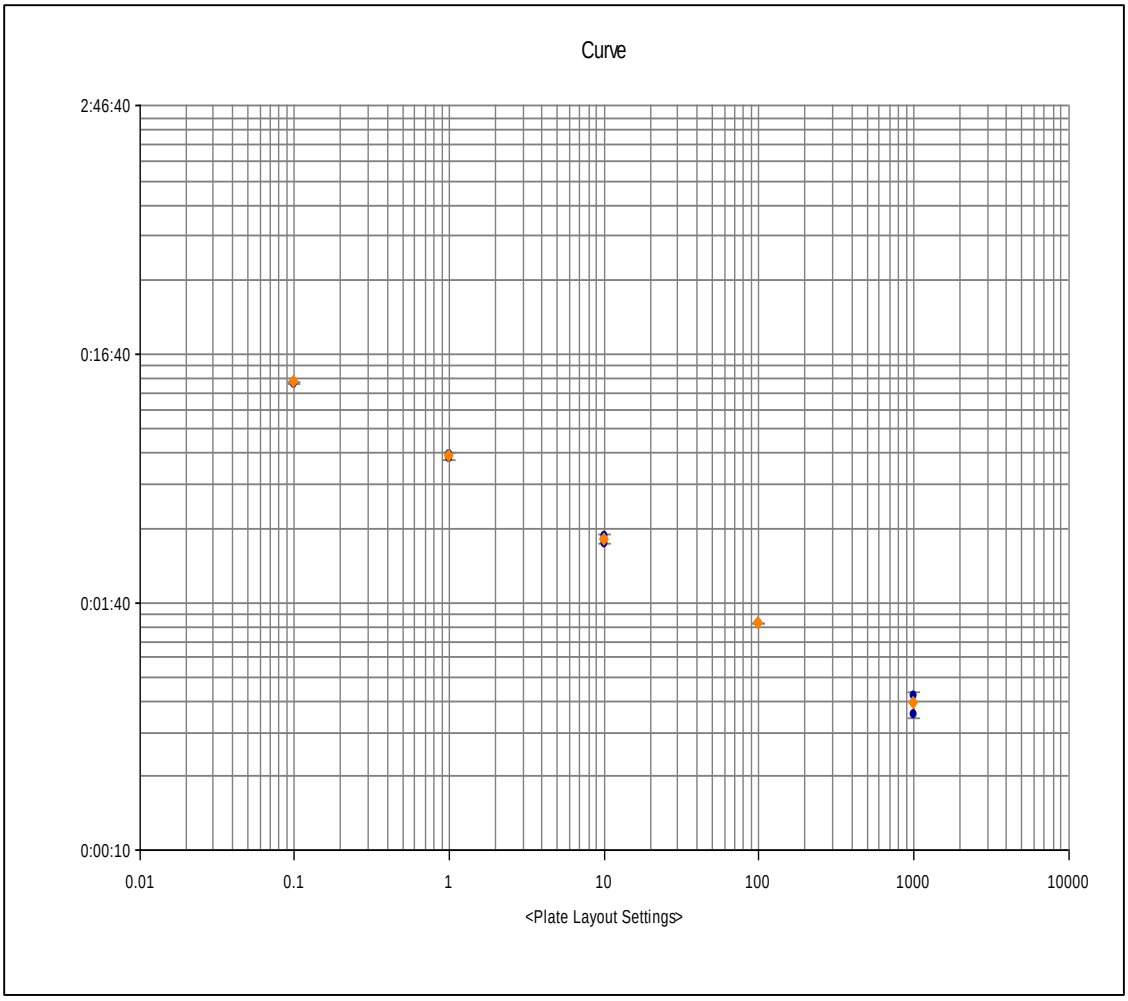

Fig.19. Standard curve produced when endotoxin concentration in ruminal fluid (diluted 1:10 000) was quantified using a kinetic chromogenic LAL test. The concentration of standard endotoxin ranged from 0.1 to 1000 EU/mL as shown on the x-axis. The regression equation of the graph was: Log(Y)=-0.327 x Log(X) +5.57. R^2=1.

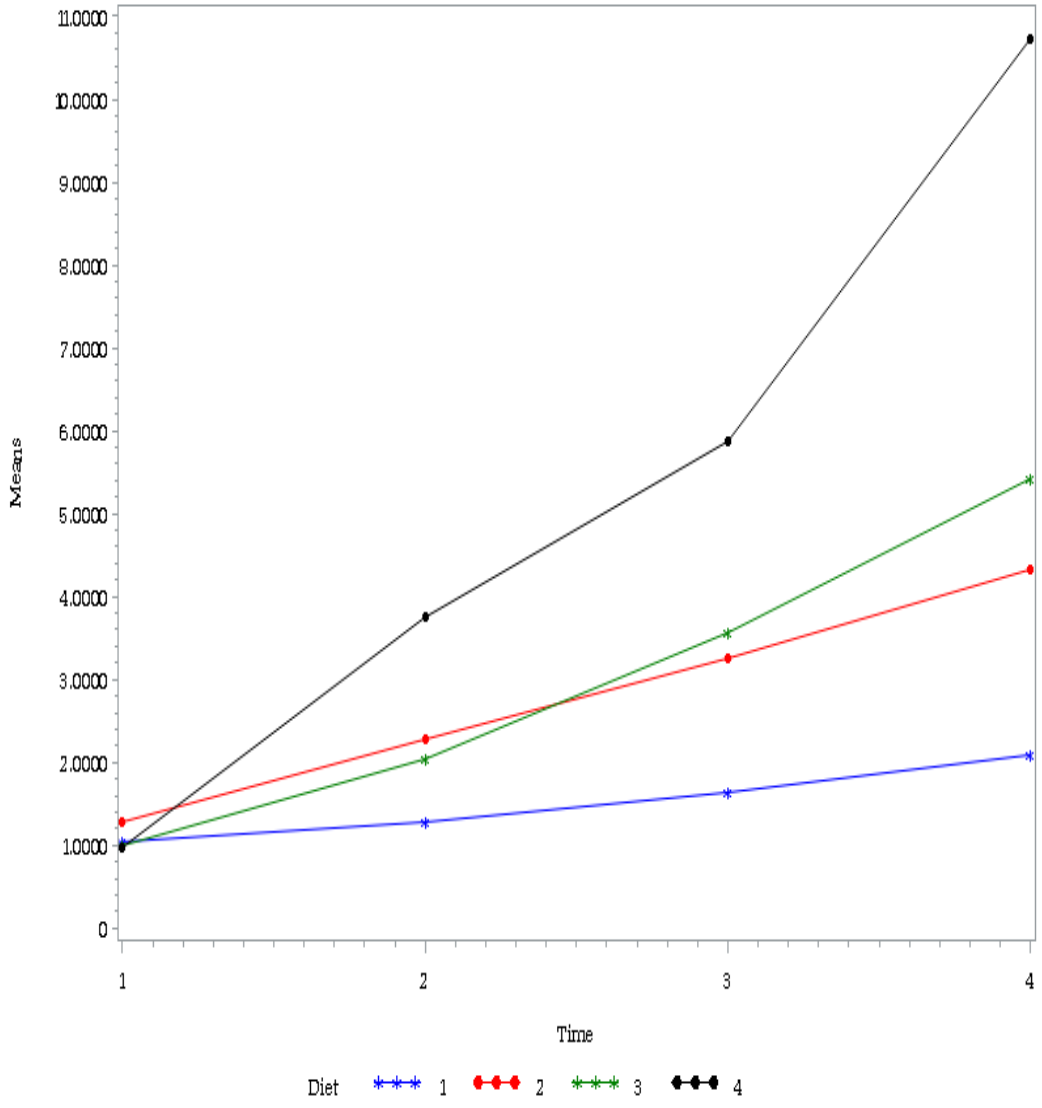

Fig.20. Ruminal fluid endotoxin concentration among the four diet groups over a 21 day period. Diet 1= Control diet group, Diet 2= 40%CHO group, Diet 3= 60%CHO group, Diet 4= 80%CHO group. Time 1= day 0, Time 2= day 7, Time 3 =day 14, Time 4= day 21. Ruminal fluid was diluted 1:10 000.

Plasma endotoxin concentration

The mean endotoxin concentration in the plasma was depended on diet and time (both $P<0.0001$).There was a significant interaction between diet and time ($P=<0.0001$). The four groups did not have similar baseline endotoxin concentrations in their plasma ($P=0.0013$). The mean plasma endotoxin concentration was different among the groups on days 7, 14, and 21($P<0.0001$ for all). The control and the 40%CHO diets did not significantly affect the concentration of endotoxin in plasma ($P=0.997$ and $P=0.224$, respectively). The 60%CHO and 80%CHO diets significantly increased the concentration of endotoxin in plasma over the 21 day period ($P=0.02$ and $P<0.0001$).

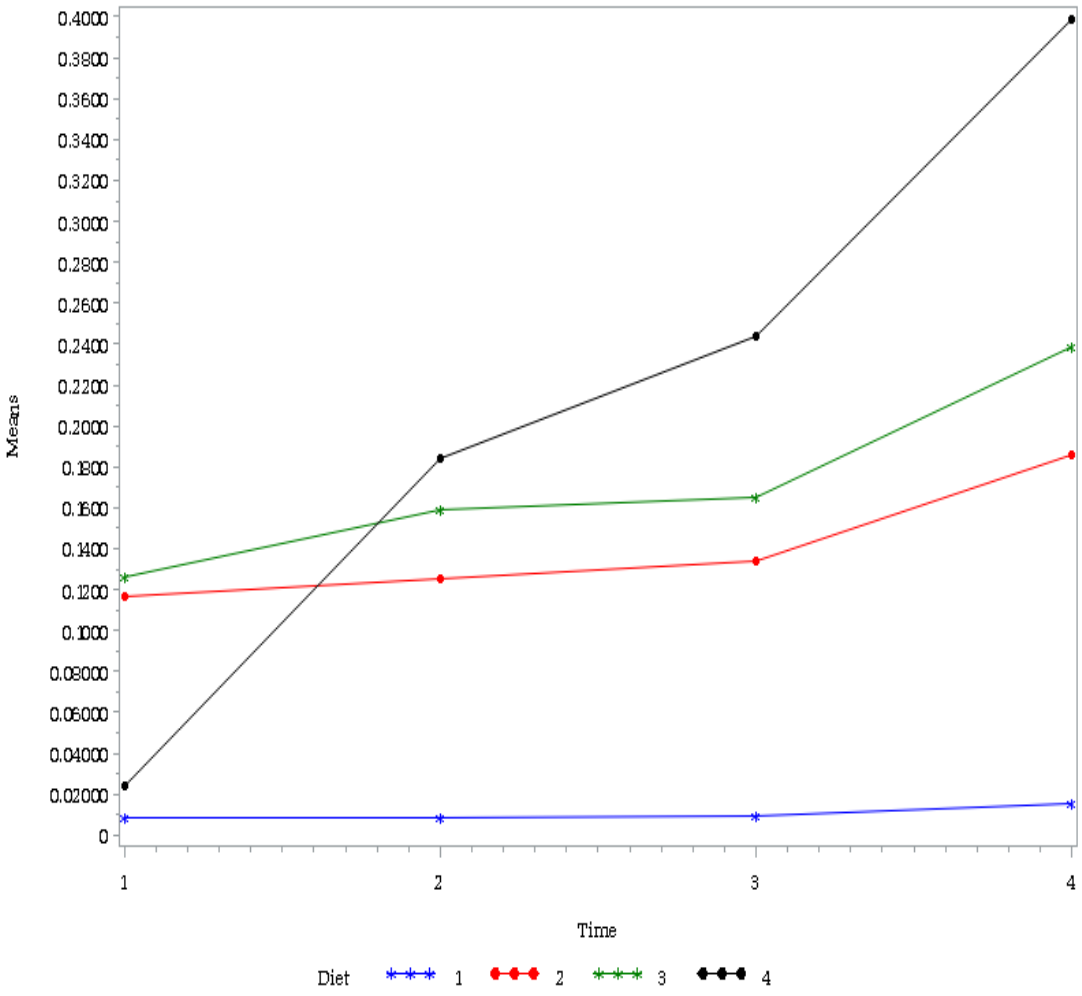

Fig.21. Plasma endotoxin concentration among the four diet groups over a 21 day period. Diet 1= Control diet group, Diet 2= 40%CHO group, Diet 3= 60%CHO group, Diet 4= 80%CHO group. Time 1= day 0, Time 2= day 7, Time 3 =day 14, Time 4= day 21. Ruminal fluid was diluted 1:40.

Relationship between ruminal fluid and plasma endotoxin concentration

There was no correlation between ruminal fluid and plasma endotoxin concentrations on day 0 ($r= 0.0733$, $P=0.690$). The concentrations of endotoxin in ruminal fluid and plasma were significantly correlated on days 7, 14, and 21 ($r=0.45$, $P=0.0099$; $r=0.672$, $P<0.0001$; $r=0.488$, $P=0.0046$).

Biogenic amines in ruminal fluid

Methylamine

Both time and diet affect the concentration of methylamine in the ruminal fluid ($P<0.001$ and $P=0.001$, respectively). The 40%CHO group had a mean ruminal fluid concentration of methylamine different from the control group ($P=0.005$). The other two groups were not different from the controls ($P=0.304$ for control vs. 60%CHO group and $P= 0.387$ for control vs. 80%CHO group). Day 0 was different from all the other day for all the groups ($P<0.001$).

Piperidine

Both time and diet affect the concentration of piperidine in the ruminal fluid ($P<0.001$). There were significant interaction between time and diet ($P<0.001$). The mean piperidine concentration was different from all the other three groups on day 7, 14 and 21 ($P<0.001$). The mean piperidine concentration was different among the three groups on days 7 and 14. The mean piperidine concentration was different from controls on day 21 in the 80%CHO group ($P=0.002$).

Putrescine

Both time and diet affect the concentration of putrescine in the ruminal fluid ($P<0.001$). There were significant interaction between time and diet ($P<0.001$). The mean putrescine concentration for the 80%CHO group was significantly different from the mean concentration in the control group for all the days ($P<0.001$). Day 0 was different from all the other days for all the groups ($P<0.001$).

Cadaverine

Both time and diet affect the concentration of cadaverine in the ruminal fluid ($P<0.001$). There was a significant interaction between time and diet ($P<0.001$). The mean cadaverine concentration for the 80%CHO group was significantly different from the mean concentration in the control group for all the days ($P<0.001$ for days 14 and 21 and $P=0.046$ for day 7).

Tyramine

Both time and diet affect the concentration of tyramine in the ruminal fluid ($P<0.001$). There was a significant interaction between time and diet ($P<0.001$). The mean concentration of tyramine was significantly different from the control in all the groups and on all the days ($P<0.001$).

Figure 22 is a chromatogram of one the samples. Figures 23-27 show the average concentration of biogenic amines in ruminal fluid over the 21 day study period.

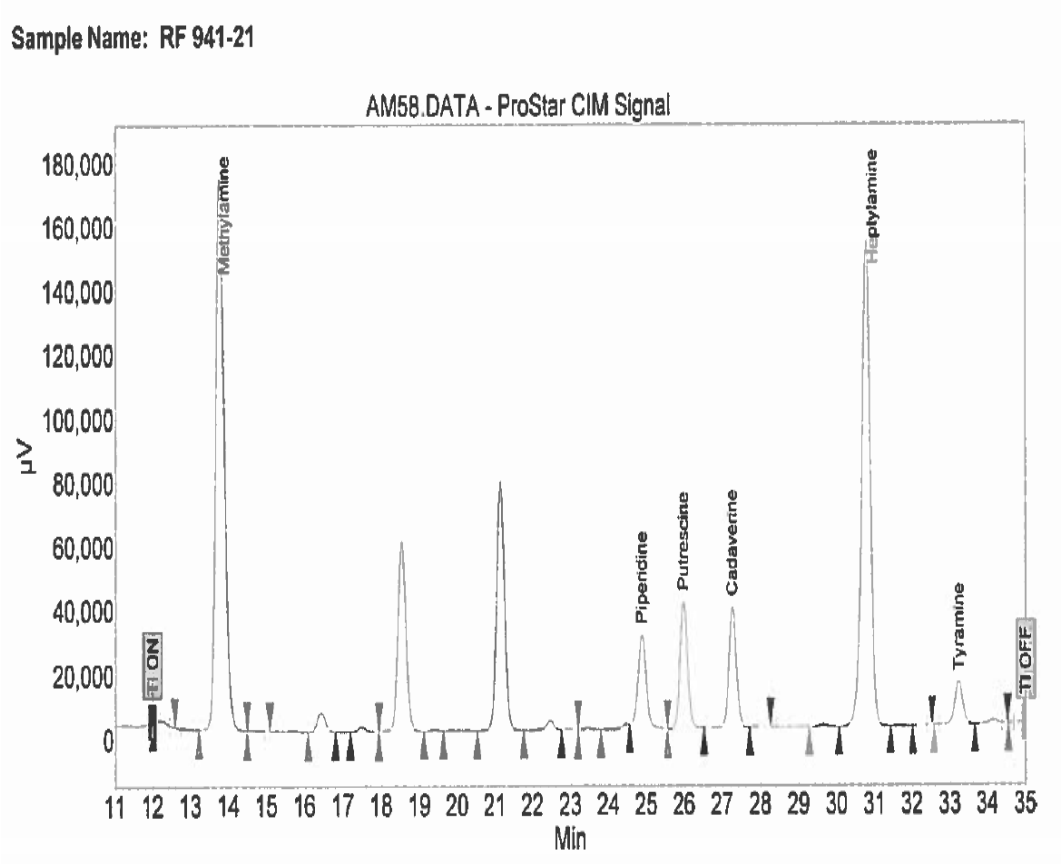

Fig. 22. Chromatogram showing quantified amines in a ruminal fluid sample. There were two unidentified peaks with retention time of 18.7 mins and 21.3 mins

Methylamine

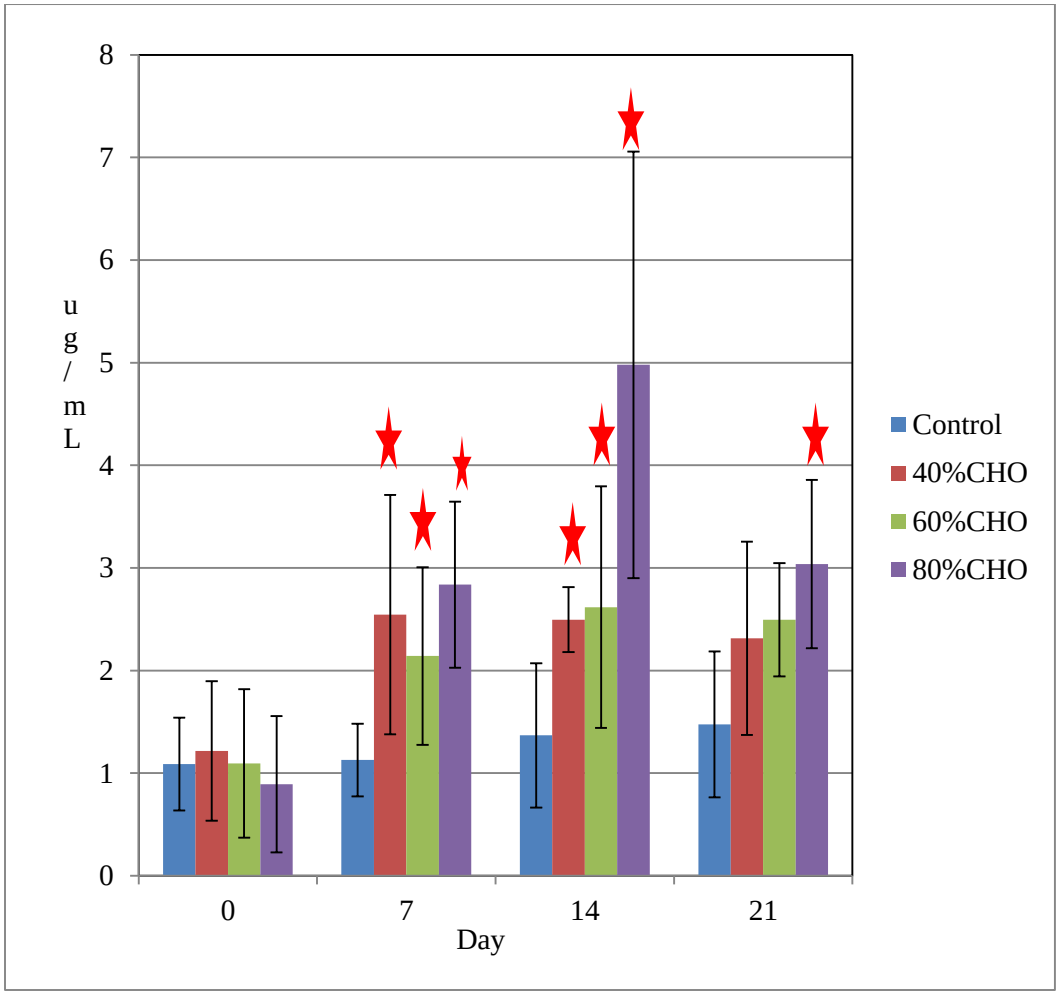

Fig. 23. Concentration of methylamine in ruminal fluid over 21 days. Biogenic amines were derivatized with dansyl chloride and heptylamine was the internal standard. Fluorescence detection was used with excitation and emission at 337 and 492nm respectively. There were 8 animals/group. Group means were compared to control.An asterisk indicates a mean concentration that is significantly different from control.

Piperidine

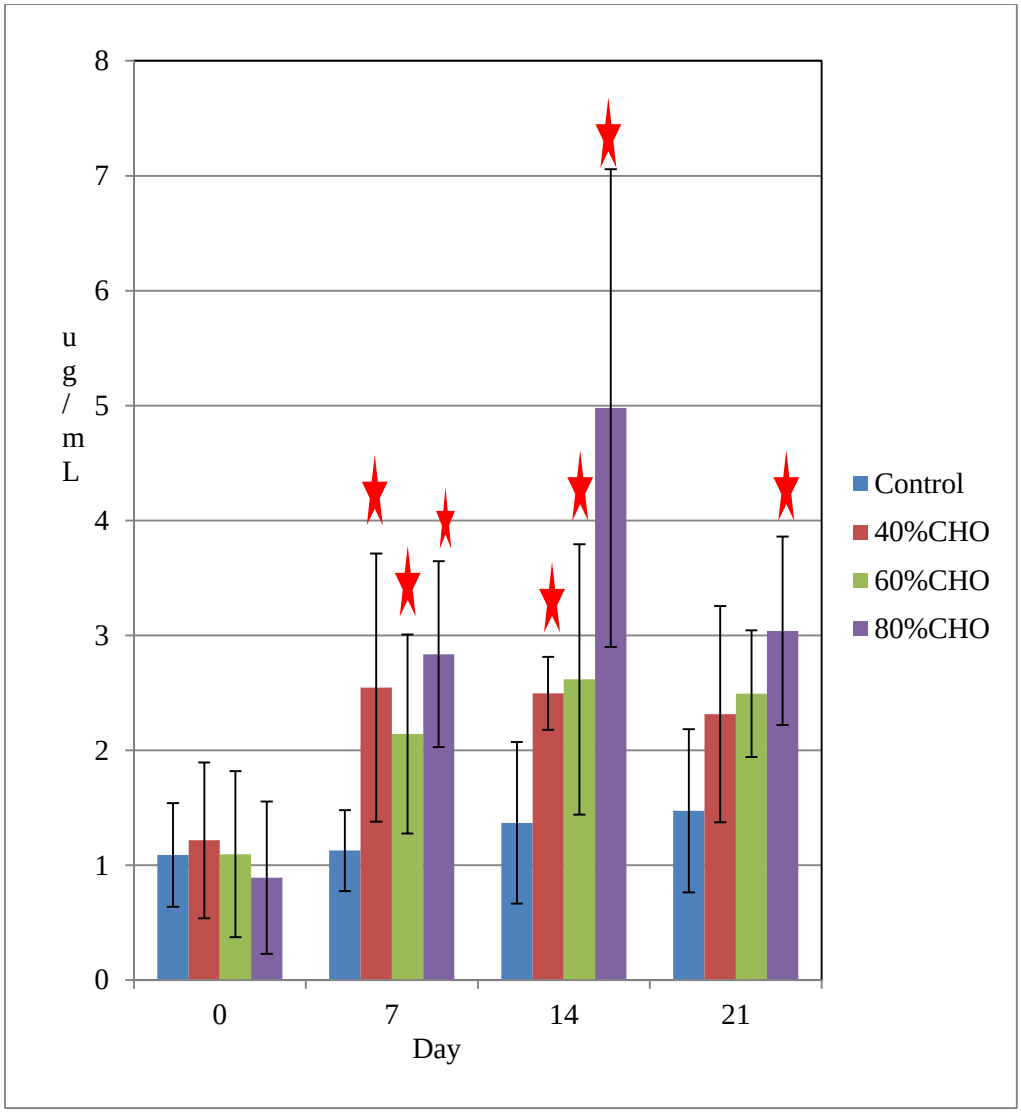

Fig. 24. Concentration of piperidine in ruminal fluid over 21 days. Biogenic amines were derivatized with dansyl chloride and heptylamine was the internal standard. Fluorescence detection was used with excitation and emission at 337 and 492nm respectively. There were 8 animals/group. Group means were compared to control.An asterisk indicates a mean concentration that is significantly different from control.

Putrescine

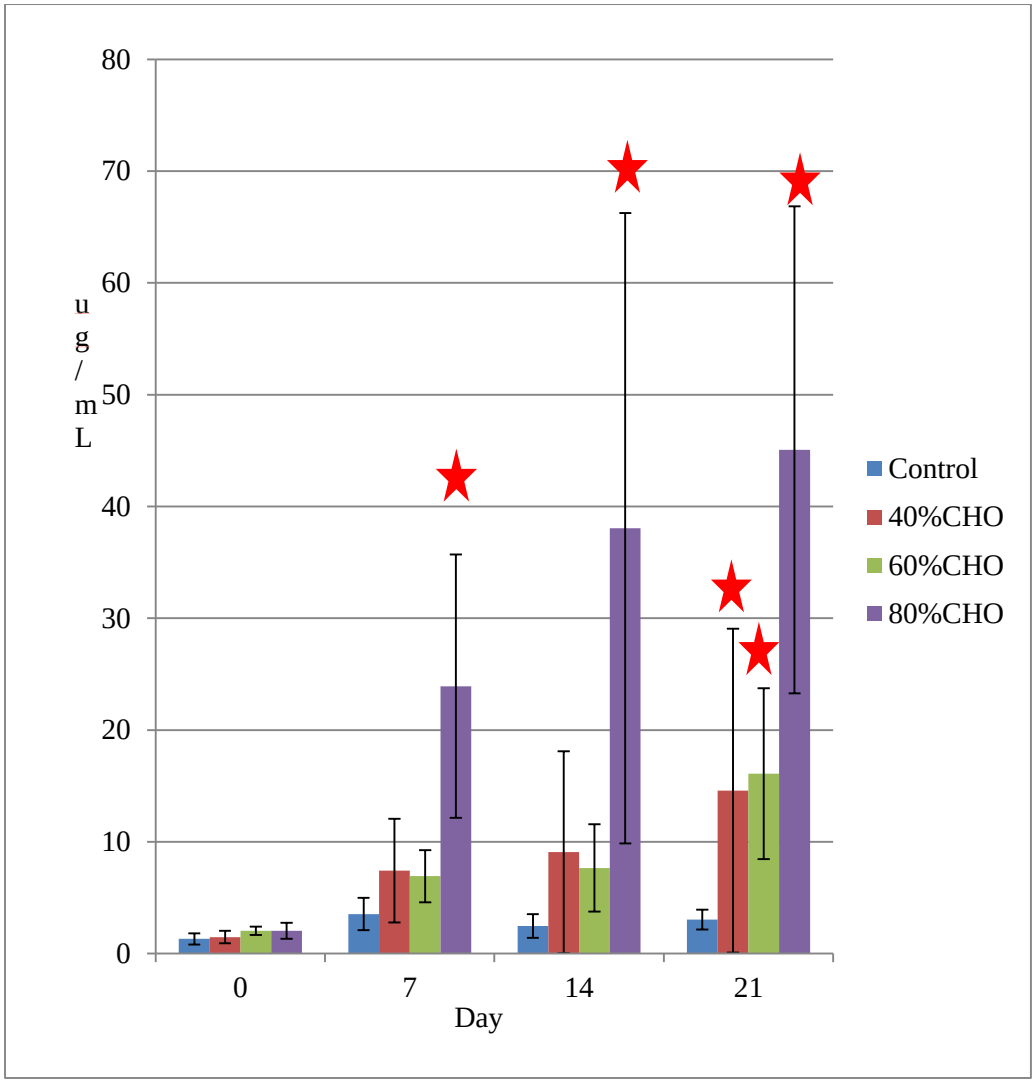

Fig. 25. Concentration of putrescine in ruminal fluid over 21 days. Biogenic amines were derivatized with dansyl chloride and heptylamine was the internal standard. Fluorescence detection was used with excitation and emission at 337 and 492nm respectively. There were 8 animals/group. Group means were compared to control.An asterisk indicates a mean concentration that is significantly different from control.

Cadaverine

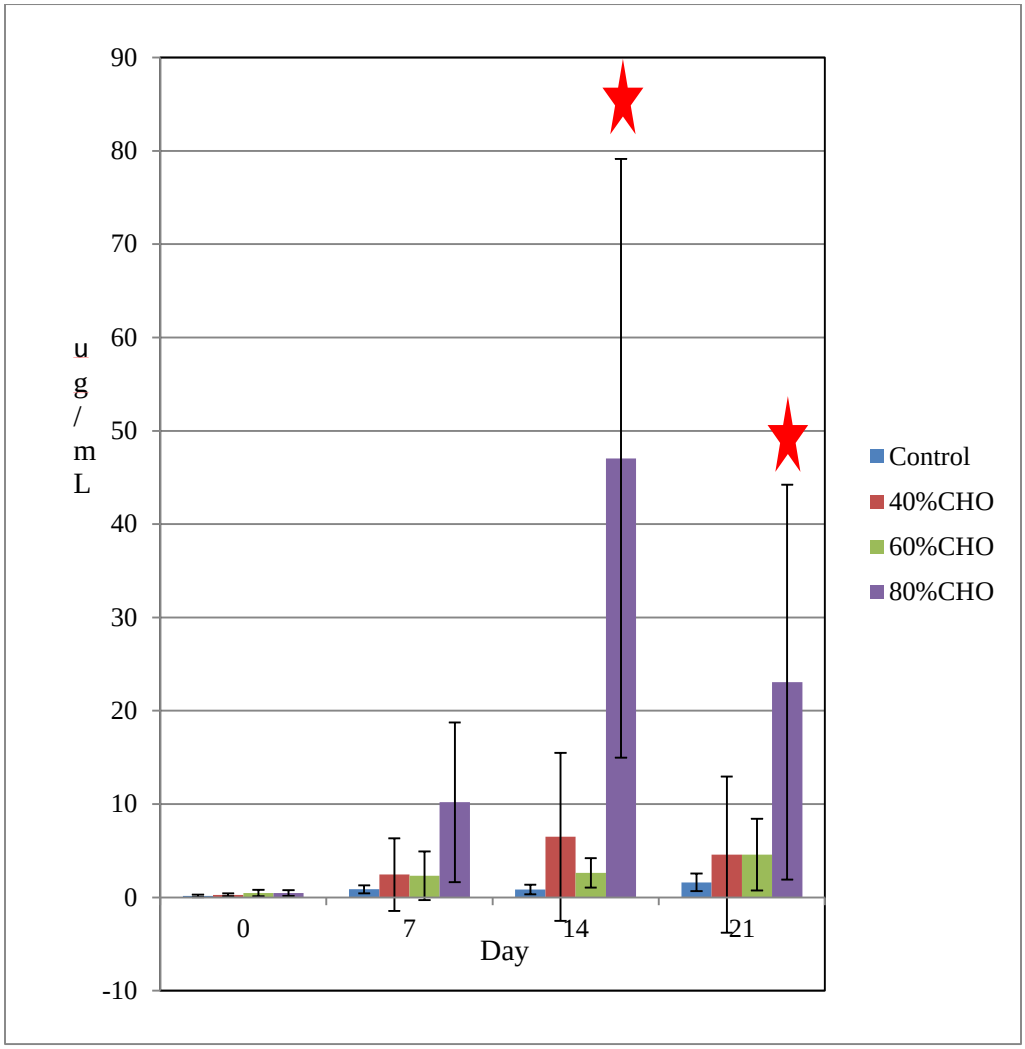

Fig. 26. Concentration of cadaverine in ruminal fluid over 21 days. Biogenic amines were derivatized with dansyl chloride and heptylamine was the internal standard. Fluorescence detection was used with excitation and emission at 337 and 492nm respectively. There were 8 animals/group. Group means were compared to control.An asterisk indicates a mean concentration that is significantly different from control.

Tyramine

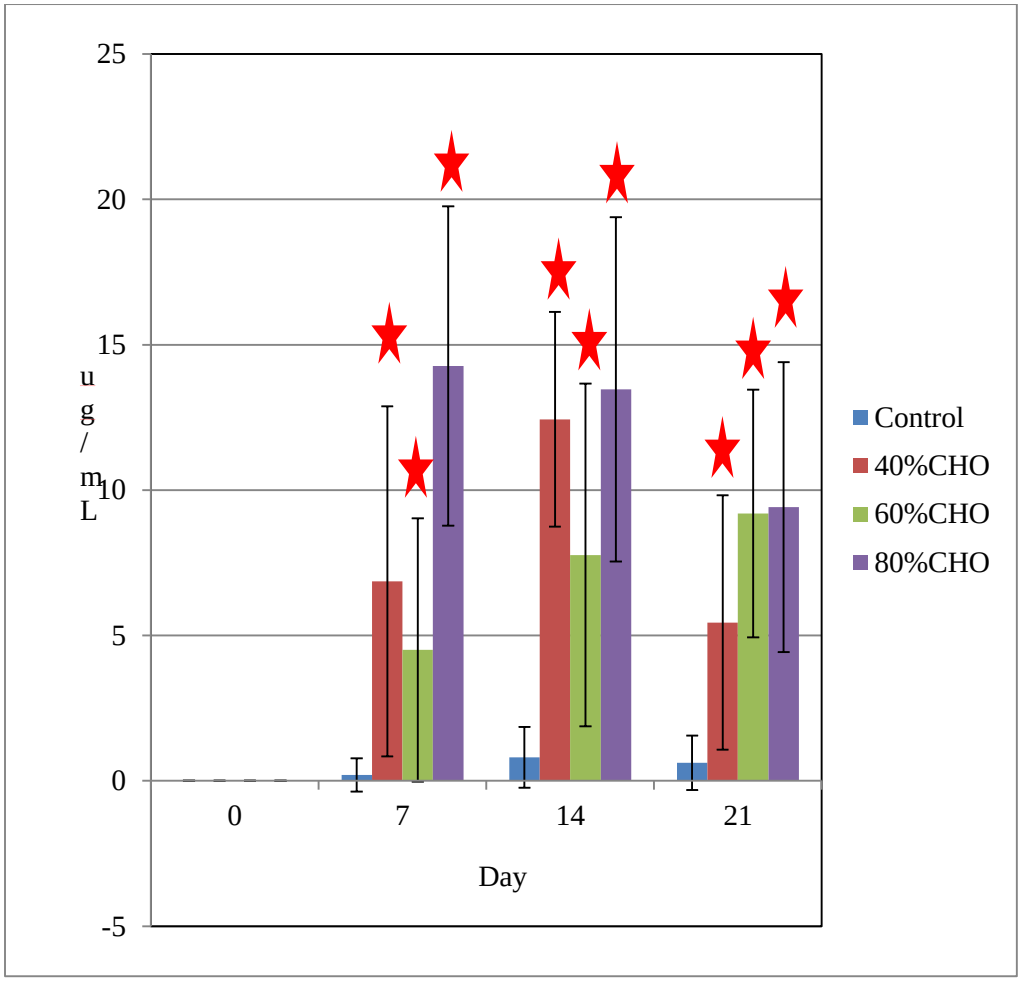

Fig. 27. Concentration of tyramine in ruminal fluid over 21 days. Biogenic amines were derivatized with dansyl chloride and heptylamine was the internal standard. Fluorescence detection was used with excitation and emission at 337 and 492nm respectively. There were 8 animals/group. Group means were compared to control.An asterisk indicates a mean concentration that is significantly different from control.

Discussion

None of the animals exhibited any clinical signs of disease throughout the study period. Body temperature was monitored by ruminal boluses and there were no differences among the group. Ruminal temperature remained ≤ 39° C in all groups during the study period. The absence of clinical disease was a favorable outcome because it eliminated a potential confounder of inflammation in our study subjects.

There were significant differences in ruminal pH among the groups. Ruminal pH tended to decrease with time in animals receiving 60% and 80% fermentable carbohydrates. Up to 30% of animals per group developed SARA in the 60%CHO and the 80%CHO group. This result provided supporting evidence of SARA in the two groups. Interestingly, significant levels of SARA developed in the animals despite the fact that their diets contained monensin and thus the efficacy of monensin in preventing SARA. Ionophores such as monensin and lasalosid are used to increase feed efficiency in the US feedlot cattle by reducing hydrogen and formic acid producers which in turn results in increased propionate production.[24] Ionophores also reduce lactate producing bacteria.[25] Monensin was successfully used to increase ruminal pH in beef cattle receiving high grain diets and in transition dairy cows.[26,27] However, in one study, monensin was not efficacious in raising ruminal pH in SARA induced dairy cows.[28] The inconsistency of monensin as a substance that increases ruminal pH could be due to differences in the concentrations of ruminal lactic acid in the studies.[28] Ruminal lactic acid concentration was reported to be above 5mM in most studies that reported the efficacy of monensin in increasing ruminal pH whereas a concentration of less than 1mM was reported in studies that reported monensin not being efficacious.[29,30] These studies suggest that monensin likely is efficacious against acute ruminal acidosis but its efficacy for the prevention of SARA might be equivocal

The concentration of endotoxin increased steadily in animals receiving 60% and 80% concentrate both in blood and ruminal fluid. The elevated levels of endotoxin suggest that there was translocation of endotoxin from the gut into systemic circulation and the amount that translocated increased with time. There was a negative correlation between ruminal fluid pH and endotoxin levels in both blood and ruminal fluid. This correlation between ruminal fluid pH and endotoxin levels in blood provides supporting evidence that reduced ruminal pH observed during episodes of SARA results in increased free endotoxin that may translocate into systemic circulation.

Molecular based techniques continue to improve our understanding of ruminal microbiota. PCR techniques have been developed to monitor changes to specific bacteria in cannulated cattle in controlled experiments.[10,11] However, due to the complexity of the rumen, and the variation in cattle breeds, diets and environment, more work is necessary to assess the effect of various production systems. In addition, the use of cannulated animals often involves small samples sizes and some level of an alteration of the environment depending on the experimental design. In the current study we have utilized qRT-PCR to monitor relative changes in ruminal bacteria in steers receiving varying amounts of fermentable carbohydrates in their diets.

Among the six bacteria that were investigated, *Megaspahera elsdenii* had the most obvious pattern in the group that was receiving an 80% fermentable carbohydrate diet. Quantitative real-time PCR analysis displayed a 150-fold increase in the *Megasphaera elsdenii* population in the group that was receiving 805 fermentable carbohydrates on day 7 and 138-fold increase on day 14. There was a 21-fold increase in the same group on day 21. The population of *Megasphaera elsdenii* also increased in the group that was receiving a 60% fermentable carbohydrate diet. The fold change increased on day 7and 14, and was spastically different on day 21. These results are consistent with the results of a previous study in which bacterial population changes were monitored for steers receiving step up diets.[10] *Megasphaera elsdenii* is one of the most widely studied rumen organisms[31] and is known to utilize the lactic acid produced within the rumen to

help prevent lactic acid accumulation and ruminal acidosis.[32] The increase in the *Megasphaera elsdenii* population is a mechanism of maintaining ruminal pH by utilizing the increasing lactic acid produced in the rumen when highly digestible carbohydrates are fed. The 21-fold decrease in the *Megasphaera elsdenii* population in the 80%CHO group on day 21 may be due to the decrease in ruminal pH beyond the survival limits of the organism or yet some unidentified mechanism.

The fold change in the population of *Fibrobacter succinogenes* was not statistically different among the four groups. However, there was a numerical decrease in *Fibrobacter succinogenes* population in the 80%CHO group. In addition, the magnitude of the increase in fold change gradually decreased from day 7 to day 21 in the 60%CHO, 40%CHO group and the controls. *Fibrobacter succinogenes* is a fibrolytic bacterium that digests fiber[11] and is predominantly present in diets high in fiber. Therefore, *Fibrobacter* populations were expected to drop in the 60%CHO group and the 80%CHO. The lack of statistical significance may be due to the sensitivity of the organism to pH change in all groups. All groups including the controls received highly fermentable carbohydrates that altered ruminal pH. The population of *Fibrobacter succinogenes* also modestly decreased in the hay only group in an experiment in which when step-up diets were fed to the treatment group.[10] However, the fold change decrease in *Fibrobacter succinogenes* population was statistically different between control and the treatment group in that study.

There was a numerical increase in the population of *Streptococcus bovis* in all the groups on day 7 and 14, and a population decrease on day 21 all groups except the controls. *Streptococcus bovis* is a facultative anaerobe and is known to predominate during lactic acidosis.[3,33,34] The lack of significant change in the population of *Streptoccus bovis* in the current study is consistent with previous reports[3,10] and suggests that although the average pH changed in the steers over time, the *Streptococcus bovis* population was still controlled and steers did not develop lactic acidosis. This

finding is supported by the fact that all animals remained clinically healthy during the entire study period. Lactic acidosis is an acute multisystemic disease whose overt clinical signs would have been expected to be observed during the study.

There were significant decreases in the population of *Ruminococcus flavefaciens* in the group that was receiving an 80% highly digestible carbohydrate diet on day 7 and 14.The population of *Ruminococcus flavefaciens* increased over time in the 60%CHO group but the increase was not statistically different from the controls. Like *Fibrobacter succinogenes*, *Ruminococcus flavefaciens* is fibrolytic and its population is expected to decrease when highly digestible carbohydrates are fed.[11] The decrease in population of *Ruminococcus flavefaciens* was observed on day 7 and 14 in the 80%CHO group. This finding suggests that *Fibrobacter succinogenes* might be able to adapt to decreases in pH better than *Ruminococcus flavefaciens*.

The population of *Prevotella ruminocola* increased in all groups but was only statistically different between control and the 80%CHO group on day 7. The observed increase in the population of *Prevotella ruminocola* is consistent with previous studies.[10,11] This finding suggests that *Prevotella ruminocola* is able to adapt well to moderate decreases in pH.

There were no significant changes in the population of *Lactobacillus acidophilus* in all groups. Like *Streptococcus bovis*, *Lactobacillus acidophilus* is lactilytic and thrives when ruminal pH is low.[15] In a study in which subacute ruminal acidosis was induced in dairy cows, the population of *Lactobacillus* spp. was significantly increased in the treatment group compared to controls. Population changes for *Streptococcus bovis* approached significance in that study. In the same study, there was a difference in the amount of lactic acid between control and the treatment group. The increase in lactic acid in the treatment group in that study may be responsible for the lack of agreement with the current study.

The interaction between diet and inflammation has received some considerable investigation and reviews in the past few years.[35,36,37,38] Highly fermentable carbohydrates have been associated with increased acute phase proteins in blood and increased expression of TNF-α, IL-1β and IL-6 in the liver and rumen epithelium of cows and steers that had experimentally induced SARA.[4,36,39] The current study focused on understanding the interaction between highly digestible carbohydrates and the innate immune system in the pulmonary system and in the systemic circulation by measuring cytokine expression in BALF and blood. We specifically performed qRT-PCR to quantify specific mRNA of the cytokines. The expression of cytokine mRNA was significantly different among the four groups with IL-1β and TNF-α demonstrating a similar pattern in blood and BALF. IL-1β mRNA was upregulated in both the 60%CHO group and the 80%CHO group on day 7 and 14. By day 21, IL-1β was down regulated in the 60%CHO and the 80%CHO groups. IL-1β is produced by macrophages when stimulated by CD14 and TLR4.[40] CD14 and TLR4 are pattern recognition receptors (PRRs) that recognize pathogen associated molecular patterns (PAMPs) such as lipopolysaccharide/endotoxin.[40] IL-1β acts on vascular endothelial cells to make them adhesive for neutrophils. IL-1β also acts on other macrophages or monocytes to stimulate synthesis of nitric oxide synthase 2 (NOS2) and cyclooxygenase-2 (COX-2) to enhance inflammation. The up-regulation of IL-1β in the 60%CHO group and the 80%CHO in the current study suggests that the two diets were associated with pulmonary or systemic circulation. The down-regulation of IL-1β in the same groups of animals suggests that either inflammation had resolved by that time or the innate immune system became tolerant to the PAMPs or alarmins. The concentration of endotoxin in circulation increased from day 0 to 21, suggesting that the immune system continued to be challenged. If this assumption is correct then the down-regulation of IL-1β is likely to be a result of immune tolerance. Endotoxin tolerance is a condition in which cells that are exposed to low concentrations of endotoxin enter into a transient unresponsive state and are unable to respond to further challenges with endotoxin in the same magnitude.[41] Endotoxin tolerance has not been reported in cattle. There are mixed views about

the role of endotoxin tolerance in the species (rabbits, mice, rates, and humans) in which it has been studied.[41] TNF-α, IL-6, and to some extent IL-1β, have been consistently found to be down-regulated in most studies where endotoxin tolerance was induced by dosing endotoxin.[41] In one study, LPS-resistant mice were remarkedly hyporesponsive to the colony stimulating factor effects of infection not only by gram negative *E. coli* but also by gram positive bacteria such as *Staphylococcus aureus*.[42] Cattle might be different from the studied species but the down regulation of both IL-1β and TNF-α in the face of increasing endotoxin levels suggest that endotoxin tolerance might also occur in cattle. However, it cannot be deduced from the current study if endotoxin tolerance is beneficial or deleterious to the health of cattle. If endotoxin tolerance is beneficial, it implies that cattle are able to adapt to constant challenge with endotoxin and prevent overproduction of pro- inflammatory cytokines. This in part explains the low morbidity in finishing feedlot cattle that receive approximately 90% fermentable carbohydrates per dry matter basis in their diets. If endotoxin tolerance is deleterious, it implies that cattle may become more susceptible to infection including respiratory pathogens early in the feeding period when carbohydrates are introduced in high amounts. Such a scenario may explain some occurrence of high BRD morbidity during the late finishing period although this is not a common occurrence. TNF-α is also proinflammatory like IL-1β. Like IL-1β, it was also up-regulated in the 60%CHO and the 80% CHO group on day 7 and 14 in both blood and BALF. It was down-regulated by day 21 in blood. There was also a significant correlation in the expression of IL-1β and TNF-α between blood and BALF.

There were also changes in the expression IL-8, IL-10, and TGFβ1 in both blood and BALF. However, there were no significant correlations of fold changes of these cytokines between blood and BALF. The differences may be due to the challenge of the respiratory system pathogens in the environment. In addition, eructated gases might also contain PAMPs that interact with sentinel cells in the pulmonary system. The expression of TGFβ1 in blood did not significantly

change among the four diet groups during the study period. The lack of significant change in the expression of TGFB1 suggests either a dysregulation or early adaptation by the sentinel cells.

The concentration of biogenic amines in the ruminal fluid increased after day 0 in all groups except the controls. Methylamine was different from the other biogenic amines tested because it only increased on day 7 and 14 in the group that was receiving 40% fermentable carbohydrates. The group that was receiving 80% fermentable carbohydrates had consistently higher biogenic amine concentration that the control on all the days except day 0. These results are consistent with the previous study where biogenic amines were increased in SARA induced cows.[15] To our knowledge, this is the first study to show the increase in concentration of piperidine and cadaverine in ruminal fluid. In a previous study, it was reported that the most common biogenic amines were methylamine, tryptamine, putrescine, histamine and tyramine.[15] The difference between the current study and the study by Wang 2013 could be caused by the different methodologies. Wang 2013 used UV detection and in this study, we used fluorescence detection. We initially intended to test histamine concentration but it was found to be unstable thereby making quantification unreliable.

Conclusion

The results of this current study suggest that receiving/preconditioning diets are associated with decreased ruminal pH, increased endotoxin levels in the rumen blood, changes in ruminal bacteria populations, and changes in expression of inflammatory cytokines. In addition the results also suggest that there might be endotoxin tolerance in cattle receiving higher amounts of highly digestible carbohydrates. Further studies are necessary to investigate the importance of endotoxin tolerance in cattle.

References

1. Cooper R, Klopfenstein T. 2006. Effects of Rumensin and feed intake variation on ruminal pH. Update on Rumensin / Tylan /Micotyl for the Professional Feedlot Consultant. Elanco Animal Health, Greenfield, IN.

2. Gozho GN, Plaizier JC, Krause DO, Kennedy AD, Wittenberg KM. Subacute Ruminal Acidosis Induces Ruminal Lipopolysaccharide Endotoxin Release and Triggers an Inflammatory Response. *J. Dairy Sci*.2005; 88:1399–1403.

3. Nagaraja TG,Titgemeyer EC. Ruminal acidosis in beef cattle: The current microbial and nutritional outlook.*J. Dairy.Sci* (E Suppl)2007; 90:E17-E38

4. Khafipour E; Krause DO, Plaizier JC. A grain-based diet subacute ruminal acidosis challenge causes translocation of lipopolysaccharide and triggers inflammation. *J. Dairy Sci.* 2009a; 92:1060-1070.

5. Horadagoda NU, Knox KM, Gibbs HA, Reid SW, Horadagoda A, Edwards SE, Eckersall PD. Acute phase proteins in cattle: Discrimination between acute and chronic inflammation. *Vet. Rec* 1999; 144:437–441.

6. Plaizier JC, Krause DO, Gozho GN, McBride BW.Subacute ruminal acidosis in dairy cows: The physiological causes, incidence and consequences. *The Veterinary Journal* 2009; 176: 21–31.

7. Kleen JL, Hooijer GA, Rehage J, Noordhuizen PTM. Subacute Ruminal Acidosis (SARA): a Review. *J. Vet. Med. A* 2003; 50: 406–414.

8. Khafipour E , Krause DO , Plaizier JC. Alfalfa pellet-induced subacute ruminal acidosis in dairy cows increases bacterial endotoxin in the rumen without causing inflammation. *J. Dairy Sci.* 2009b; 92 :1712–1724.

9. Steele MA, Croom J, Kahler M, ALZahal O, Hook SE, Plaizier K, McBride BW. Bovine rumen epithelium undergoes rapid structural adaptations during grain-induced subacute ruminal acidosis. American Journal of Physiology –Regulatory, Integrative and Comparative Physiology 2011; 300: R1515–R1523.

10. Fernando SC, Purvis 11 HT, Najar FZ, Sukharnikov LO, Krehbiel CR, Nagaraja TG, Roc BA, DeSilva U. Rumen microbial population dynamics during adaptation to a high-grain diet. *Appl Environmental Microbiology*.2010;76:7482-7490.

11. Tajima K, Aminov RI, Nagamine T, Matsui H, Nakamura M, Benno Y. Diet-dependent shifts in the bacterial population of the rumen revealed with real-time PCR. *Applied and Environmental Microbiology* 2001; 67: 2766-2774.

12. Goad DW, Goad CL, Nagaraja TG. Ruminal microbial and fermentative changes associated with experimentally induced subacute acidosis in steers. *J. Anim. Sci* 1998; 76: 234-241.

13. Khafipour E, Plaizier JC, Aikman PC, Krause DO. Population structure of rumen *Escherichia coli* associated with subacute ruminal acidosis (SARA) in dairy cattle. *J. Dairy Sci.*2011; 94:351–360

14. Fusi E, Rossi L, Rebucci R, Cheli F, et al. Administration of biogenic amines to Siaanen kids : effects on growth performance, meat quality and gut histology. *Small ruminant Res* 2004; 53: 1-7.

15. Wang DS, Zhang RY, Zhu WY, Mao SY. Effects of subacute ruminal acidosis challenges on fermentation and biogenic amines in the rumen of dairy cows. *Livestock Science* 2013; 155:262–272

16. Colman E, Fokkink WB, Craninx M, Newbold JR, De Baets B, Fievez V. Effect of induction of subacute ruminal acidosis on milk fat profile and rumen parameters. *J Dairy Sci* 2010; 93: 4759-4773.

17. Lofgreen GP. Nutrition and management of stressed beef calves.*Vet.Clin.North.Am*.1983; 5:87-101

18. Mwansa PB, Makarechian M, Berg RT. The effect of level of concentrate in feedlot diets on the health status of beef calves. *Can Vet J* 1992; 33: 665-668.

19. Liu L, Gao Z, Xia C, Xu Y, Ma Z, Dong C, Li B. Comparative Study of Trans-Oral and Trans-Tracheal Intratracheal Instillations in a Murine Model of Acute Lung Injury. *The Anatomical Record* 2012; 295:1513–1519.

20. Garret EF, Pereira MN, Nordlund KV, et al Methods for the Detection of Subacute Ruminal Acidosis in Dairy. *Cows J Dairy Sci* 1999; 82:1170–1178.

21. Step DL, Krehbiel CR, Depra HA et al. The effects of commingling beef calves from different sources and weaning protocols during a forty- two -day receiving period on performance and bovine respiratory disease. *J Anim Sci* 2008, 86:3146-3158.

22. Burciaga-Robles LO, Step DL, Krehbiel CR, et al. Effects of exposure to calves persistently infected with bovine viral diarrhea virus type 1b and subsequent infection with Mannheimia haemolytica on clinical signs and immune variable: Model for bovine respiratory disease via viral and bacterial interaction. *J Anim Sci* 2010; 88: 2166−2178

23. Wyckoff 3rd JH, Howland JL, Scott CM, Smith RA, Confer AW. Recombinant bovine interleukin 2 enhances immunity and protection induced by Brucella abortus vaccines in cattle. *Veterinary Microbiology*. 2005; 111: 77–87.

24. Russell JB, Hino T. Regulation of lactate production in *Streptococcus bovis*: A spiraling effect that contributes to rumen acidosis 1985. *J Dairy Sci* 68:1712–1721.

25. Hutjens MF, Feed Additives. *Vet Clinics of Nor Amer. Food Anim Pract* 1991; 7(2) : 525–540.

26. Nagaraja, T. G., T. B. Avery, S. J. Galitzert, and D. L. Holman. Effect of ionophore antibiotics on experimentally induced lactic acidosis in cattle. *Am J Vet Res* 1985; 46:2444–2452.

27. Green BL, McBride BW, Sandals WD, Leslie KE, Bagg R, Dick P The impact of the monensin controlled release capsule upon subclinical acidosis in the transition dairy cow. *J Dairy Sci* 1999; 82:333–342.

28. Langlois BE, Dawson KA, Leak I, Aaron DK. Antimicrobial resistance of fecal coliforms from pigs in a herd not exposed to antimicrobial agents for 126 months. *Vet Microbiol* 1988; 18: 147–153.

29. Osborne JK, Mutsvangwa T, Alzahal O, Duffield TF, Bagg R, Dick P, Vessie G, McBride BW. Effects of Monensin on Ruminal Forage Degradability and Total Tract Diet Digestibility in Lactating Dairy Cows During Grain-Induced Subacute Ruminal Acidosis. *J Dairy Sci* 2004; 87:1840–1847.

30. Owens FN, Secrist DS, Hill WJ, Gill DR. Acidosis in cattle: A review. *J Anim Sci* 1998; 76: 275–286.

31. Russell J B, Dombrowski DB. Effect of pH on the efficiency of growth by pure cultures of rumen bacteria in continuous culture. *Appl. Environ. Microbiol* 1980; 39:604–610.

32. Counotte GHM, Prins RA, Janssen RHAM, Debie MJA. Role of *Megasphaera elsdenii* in the fermentation of lactate-2-C-13 in the rumen of dairy cattle. *Appl. Environ. Microbiol* 1981; 42:649–655.

33. Dunlop RH, Hammond PB. D-Lactic acidosis of ruminants. Ann. N. Y. Acad. Sci 1965; 119:1109–1132.

34. Slyter LL. 1976. Influence of acidosis on rumen function. *J. Anim. Sci* 1976; 43:910–929.

35. Emmanuel DGV, Madsen KL, Churchill TA, Dunn SM, Ametaj BN. Acidosis and Lipopolysaccharide from *Escherichia coli* B:055 Cause Hyperpermeability of Rumen and Colon Tissues *J. Dairy Sci*. 2007;90:5552–5557.

36. Zebeli Q, Metzler-Zebeli BU, Ametaj BN. Meta-analysis reveals threshold level of rapidly fermentable dietary concentrate that triggers systemic inflammation in cattle. *J. Dairy Sci.* 2012; 95 :2662–2672.

37. Saleem F, Ametaj BN, Bouatra S, Mandal R, Zebeli Q, DunnSM , Wishart DS. A metabolomics approach to uncover the effects of grain diets on rumen health in dairy cows *J. Dairy Sci.* 2012; 95 :6606–6623.

38. Gaylean ML, Perino LJ, Duff GC. Interaction of cattle health/immunity and nutrition. *J. Anim. Sci*.1999; 77:1120-1134.

39. Ametaj BN, Koenig KM, Dunn, Yang WZ, Zebeli Q, Beauchemin KA. Backgrounding and finishing diets are associated with inflammatory responses in feedlot steers. *J. Anim Sci* 2009; 87:1314-1320.

40. Tizard IR. 2009. Veterinary Immunology: An Introduction. 8the Ed, Saunders Elsevier

41. Mengozzi M, Ghezzi P. Cytokine down-regulation in endotoxin tolerance. *Eur. Cytokine Netw* 1993; 4 (2): 89–98.

42. Quesenberry P, Cohen H, Levin J, et al . Effects of bacterial infection and irradiation on serum colony-stimulating factor levels in tolerant and nontolerant CFI mice. *Blood* 1986; 51:229

CHAPTER V

CONCLUSIONS

Metabolism in the rumen is very complex process that involves a symbiotic relationship between the microbes and the host animal. This symbiotic relationship enables ruminants to utilize feed sources that are not digestible by mammalian enzymes. Cattle have evolved to feeding forage based diets. However, in order to optimize returns within a reasonable amount of time, concentrates are fed to cattle. The introduction of concentrates or highly fermentable carbohydrates to cattle alters rumen metabolism. The change in rumen metabolism is demonstrated by changes in bacterial populations, changes in ruminal pH, changes in proportions of volatile fatty acids and other catabolic products such as biogenic amines. Our research focused on demonstrating changes in ruminal metabolism and the animals respond to those changes.

The objectives of the first study were to assess the feasibility of performing rumenocentesis in beef cattle under typical settings and evaluate the occurrence subacute ruminal acidosis (SARA). Results of the first study demonstrated that when performed by a trained veterinarian, rumenocentesis is fairly quick, safe and easy to perform under typical beef cattle handling facilities. The ability to perform rumenocentesis without encountering adverse health outcomes is beneficial because it enables investigators to evaluate natural disease states particularly for the recently weaned light-weight calves. Cannulated animals may not be truly representative of the recently weaned, high risk calves. The results also demonstrated that SARA occurs at significant proportions, around 22%, in cattle that are fed receiving/preconditioning diets. SARA develops in receiving cattle in spite of feeding monensin and other preventive measures such as proper stocking density, good bunk management, and good feed formulation. Monensin is an ionophore that targets gram positive bacteria.[1] The most important gram-positive bacteria that thrive in low pH environment are *Streptococcus bovis* and *Lactobacillus* spp. There were no significant changes in the populations of *Streptococcus bovis* and *Lactobacillus* spp suggesting that either monensin was quite effective in controlling the populations of both bacteria or the kinetics favor other bacteria species. Although monensin is effective in the prevention of acute ruminal lactic acidosis, its efficacy against SARA is unclear. Results of the second study also revealed that protozoa viability was not a good diagnostic marker for SARA. In fact, protozoa were found to be more viable in lower pH ruminal environment than in higher pH conditions. The protozoa thrive during episodes of SARA likely because pH is not low enough to kill them; rather there is abundance of substrate for growth.[2]There was no difference in average daily gain between SARA affected animals and those without SARA. This lack of difference in average daily gain may be due to short study period. Twenty-eight days may not be enough to have detectable differences in weight. Morbidity was also not correlated with acidosis.

The objective of the second study was to evaluate potential differences in rumen environment between cattle that pulled for exhibiting clinical signs of clinical BRD and meet the treatment criteria and animals that get pulled but did not meet the treatment criteria. Indeed, ruminal pH was significantly different between animals that were pulled and met the criteria for treatment and animals that were pulled but did not meet the criteria for treatment. The median ruminal fluid pH for animals that were treated was 6.6 and the median ruminal fluid pH for the animals that were not treated was 5.59. This suggests that SARA might be a confounder for the diagnosis of BRD. We were unable to ascertain the treated animals had progressed from low ruminal pH to having anorexia, indigestion and higher ruminal pH. A study in which ruminal pH is constantly monitored is necessary to determine the progression of SARA affected animals.

The results of the third study demonstrated a lot of significant changes in groups of animals receiving varying amounts fermentable carbohydrates in their diets. Ruminal fluid pH was significantly different among the groups with groups receiving high concentrate diets having lower ruminal pH than groups having low concentrate diets. The occurrence of SARA in this study emboldened our suspicion that the current management practices including feeding monensin may not be adequate in preventing SARA. The changes in bacterial populations in this study demonstrate that SARA mainly affects gram-negative bacteria with fibrolytics like *Fibrobacter succinogenes* being downregulated while gram- negative lactilytics like *Megasphaera elsdenii* thrive. The population changes for *Megasphaera elsdenii* in this study explain, at least in part; why *Megasphaera elsdenii* is a huge target for direct fed microbes (DFM).[3] Populations of gram-positive bacteria do not appear affected by SARA.

The concentration of endotoxin steadily increased from day 0 to day 21 in both ruminal fluid and plasma of animals receiving high concentrate diets. The increase in concentration of endotoxin in both systems over time gives further support to the growing understanding that grain-induced SARA causes translocation of endotoxin into systemic circulation.[4] The absorbed endotoxin

exceeded the liver threshold for clearance. The kinetic chromogenic LAL is now a very accurate test if performed correctly. This has enabled us to quantify endotoxin in plasma without interference. Expression of cytokines also varied according to diet. High concentrate diets were associated with inflammation. There was a dramatic shift in expression of IL-1β and TNF by day 21. This dramatic down-regulation accompanied by increasing endotoxin levels suggests endotoxin tolerance. Endotoxin tolerance has not been reported in cattle and its overall effect is unclear. More studies are needed to assess if endotoxin tolerance is beneficial or deleterious to the health of cattle. This will help to answer the question of whether high concentrate diets have an effect on the occurrence of bovine respiratory disease (BRD).

References

1. Nagaraja TG, Avery TB, Galitzert SJ, Holman DL. Effect of ionophore antibiotics on experimentally induced lactic acidosis in cattle. *Am J Vet Res 1985*; 46: 2444–2452.

2. Owens FN, Goetsch AL. 1988. Ruminal Fermentaion in The Ruminant Animal: Digestive Physiology and nutrition. Prentice Hall, New Jersey.

3. Leeuw KJ, Siebrits FK, Henning PH et al. Effect of *Megasphaera esdenii* NCMB 41125 drenching on health and performance of steers fed high and low roughage diets in the feedlot. *South African J Anim Sci* 2009; 39: 337-348.

4. Khafipour E, Krause DO, Plaizier JC. A grain-based subacute ruminal acidosis challenge causes translocation of lipopolysaccharide and triggers inflammation. *J Dairy Sci 2009*; 92:1060-1070.

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