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EFFECT OF DIRECT-FED MICROBIALS AND MONENSIN ON IN VITRO RUMEN FERMENTATION

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THESIS

A thesis submitted in partial fulfillment of the requirements for the
degree of Master of Science in the
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By

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ABSTRACT OF THESIS

EFFECT OF DIRECT-FED MICROBIALS AND MONENSIN ON IN VITRO RUMEN FERMENTATION

The impact of supplying a mixed culture of lactate producing bacteria on *in-vitro* rumen fermentation of forage- (Experiment 1) and concentrate- (Experiment 2) based diets in the presence and absence of monensin was explored. In experiment 1, interactions between DFM and MON were absent ($P>0.10$) for gas production and fermentative end products. Gas production and fermentative end products were unaffected by DFM alone ($P>0.10$). Monensin decreased ($P<0.001$) CH_4 and NH_3 production, rate and total gas production, as well as total VFA concentration and molar proportions of acetate and butyrate. However, MON increased ($P<0.001$) proportions of propionate, valerate, isobutyrate and isovalerate. Independently, DFM and MON increased ($P<0.001$) end point pH. In experiment 2, DFM x MON interactions or tendencies ($P=0.07$, $P<0.01$, $P<0.01$) were present, DFM effects were abated by MON, for rate, total gas production and total VFA concentrations. Acetate:propionate ratio was decreased ($P<0.01$) with MON and was unaffected by DFM. Ammonia-N concentration was increased ($P<0.01$) by DFM and unaffected ($P=0.75$) by MON. Both DFM and MON treatment increased ($P<0.01$) ruminal pH levels. These studies suggest the effects of DFM and MON of *in-vitro* fermentation are dependent on the substrate being fermented and the observed interactions provide means for further research.

KEYWORDS: Direct-fed microbial, monensin, rumen fermentation, forage-based diet, concentrate-based diet

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CHAPTER 1: LITERATURE REVIEW

Rumen Fermentation

The microbial population of the rumen plays an important role in the nutritional well-being of cattle. Short chain fatty acids, microbial cells, methane, heat, and ammonia are major products of rumen fermentation. These products serve either as a source of energy, protein and nitrogen or represent energy loss for the animal. The balance of these products determines the efficiency at which nutrients are utilized by ruminants (Russell and Hespell, 1981). This balance is controlled by the types of microorganisms in the rumen and substrates available for microbial growth and persistence. A symbiotic relationship exists between the ruminant and the bacterial populations that survive in the rumen of that animal; the host has the ability to supply the bacteria with the nutrients it needs to survive, the bacteria in turn break down these substrates into products the host can absorb and utilize (Thornton et al., 1952). A pronounced number of organisms are present for an ample length of time to allow for bacterial degradation of the ingested food material; the large volume and slow movement of materials through the digestive tract support this. Within the rumen environment the bacterial flora are most eminent in their ability to digest cellulose, produce fatty acids, and synthesize proteins and vitamins.

The vast majority of carbohydrates consumed by ruminants are polymers of glucose presented to the animal in the form of cellulose and starch. Some diets, particularly forage-based diets, also contain large amounts of hemicellulose and pectin. In order for fermentation of these carbohydrates to occur they must first undergo hydrolysis in the rumen. The initial stage of carbohydrate degradation and fermentation

includes the attachment of bacterial species to feed particles and the disassociation of carbohydrate polymers. Amylolytic bacterial species, *Bacteroides amylophilus, Streptococcus bovis, Succinimonas amylolytica and Succinivibrio dextrinosolvens* are the four most common amylolytic bacteria found in the rumen (Baldwin and Allison, 1983). These bacteria rapidly hydrolyze starch to maltose and some glucose depending on the source of the starch and type of feed processing methods used (G.C. Fahey, 1988). The most common cellulolytic bacterial species in the rumen are *Fibrobacter succinogenes, Ruminococcus albus, and Ruminococcus flavefaciens*. These bacterial species are responsible for producing cellulase(s), the enzyme complex which catalyzes the hydrolysis of released polymers to small saccharides (Russell and Hespell, 1981), leading to degradation of these small saccharides via the Embden-Meyerhof-Parnas pathway (glycolysis;(Wallnöfer et al., 1966). The previously mentioned symbiotic relationship of the host and bacterial flora is exemplified through the digestion process of cellulose within the rumen. Cellulase is a microbial and not a mammalian enzyme; therefore in order for digestion of carbohydrate material to occur it must first be utilized by bacteria. The conversion of cellulose to glucose and then pyruvate, the intermediate through which all carbohydrates must pass before being converted to essential end products, is considered complex given the various forms (amorphous and crystalline) in which cellulose is presented. Additionally, the same bacterial species that hydrolyze cellulose are common hemicellulose degraders, attacking hemicellulose through a non-specific hydrolysis of β-1,4 xylosidic linkages (G.C. Fahey, 1988). Ruminal pectins are degraded by means of at least two enzymes, a methylesterase and a polygalacturonidase, produced by *B. succinogenes, B. ruminicola* and *B. fibrisolvens,* as well as several species of

protozoa. The pentoses produced from hemicellulose and pectin are transformed before being converted to volatile fatty acids (VFA) via the Embden-Meyerhof pathway (Baldwin and Allison, 1983).

The proportion of end product formation is dependent on the type of substrate fermented, the bacterial species involved and the rumen environment during fermentation. Numerous intermediates are formed during carbohydrate degradation, however the major fermentation end-products that accumulate within the rumen are volatile fatty acids, carbon dioxide and methane (Russell and Hespell, 1981).

Pyruvate, the resulting product of the Embden-Meyerhof pathway which carbohydrates cycle through, can be converted to acetate through two mechanisms in rumen microbes. The pyruvate-formate lyase system is the most common pathway for acetate production that produces formate and acetyl-CoenzymeA (CoA) as intermediates. An additional pathway involving pyruvate-ferredoxin oxidoreductase can produce ferredoxin, CO_2, and acetyl-CoA. Acetyl-CoA is then converted to acetate plus ATP by phosphotransacetylase and acetokinase. Propionate production from pyruvate occurs primarily through the dicarboxylic acid pathway. Within this pathway three enzymes have been identified which catalyze the conversion of pyruvate to propionate: phosphoneolpyruvate (PEP) carboxykinase, pyruvate carboxylase, and methylmalonyl-CoA carboxyltransferase (Baldwin and Allison, 1983). The bacterial species present within the rumen environment are one determining factor that controls the enzymatic process which pyruvate follows, subsequently determining the amount of propionate produced via the dicarboxylic pathway. The acrylate pathway is an additional pathway through which propionate can be produced and has been identified in *M. elsdenii* and *B.*

rumincola. Pyruvate is converted to lactate which is then converted to acrylyl-CoA and finally to propionyl-CoA, comprising approximately one-third of total propionate production (G.C. Fahey, 1988). Similarly, two pathways have been identified for the synthesis of butyrate, the primary route being a reversal of the β-oxidation pathway. Butyrate is additionally produced through a pathway that utilizes the combination of malonyl-CoA and acetyl-CoA to produce acetoacetyl-CoA. This coenzyme is then reduced to butyrate. It is thought that the main reason for this conversion to butyrate may be to oxidize reduced bacterial cofactors allowing for further fermentation (G.C. Fahey, 1988).

The majority of VFA produced are absorbed through the rumen wall into the blood stream. During absorption across the rumen epithelium small amounts of acetate are converted to ketone bodies, however most acetate is unchanged as it passes through and is transported by portal circulation to the liver. Approximately 80% of the acetate that reaches the liver is passed into the peripheral circulation, escaping oxidation (G.C. Fahey, 1988). Once absorbed from the blood, acetate is oxidized via the tricarboxylic acid cycle (TCA) or used for fatty acid synthesis. Small amounts (2-5%) of propionate are converted to lactic acid during absorption across the ruminal epithelium, while the remainder enters the blood stream as propionate (Elliot, 1980). Once propionic acid reaches the liver it is oxidized or converted to glucose. In order to enter the TCA cycle, propionyl-CoA is fixed by CO_2 to be converted to succinyl-CoA (an intermediate in the TCA cycle). The majority of butyric acid is converted to ketones during absorption through the rumen epithelium. These ketones are then oxidized in cardiac and skeletal

muscles for fatty acid synthesis in adipose and mammary gland tissue (G.C. Fahey, 1988). The butyrate that reaches the liver is rapidly metabolized by hepatic tissue.

The rate of VFA absorption is influenced by a number of factors including pH, osmolality, and type and concentration of VFA within the rumen (Bergman, 1990). The influential factor of pH can have an effect on both the absorption and the production of VFA. Dijkstra et al. (1994), along with several other authors, observed that decreasing pH levels increase fractional absorption rates of VFA. Fractional absorption rate of propionic and butyric acids were significantly higher with low initial pH levels. Additionally the fractional absorption rate of acetic acid was not significantly affected by initial pH. However the estimated absorption rate of acetate tended to decrease with an increase in pH (Dijkstra et al., 1993). Similar results were offered by MacLeod and Orskov (1984) indicating that as rumen fluid pH decreased, the proportion of acitic acid increased and that of propionic and butyric acid decreased. Only undissociated VFA are thought to diffuse through the membrane of the rumen epithelium. Once absorbed, acids in the undissociated form dissociate rapidly, delivering H^+ ions to the cell interior (Dijkstra, 1994). Increased absorption rates are potentially obtained through high supply of H^+ ions in the rumen fluid by Na^+/H^+ exchange, continually increasing as pH falls. Dijkstra et al. (1993) observed levels of VFA absorption that were not directly proportional to VFA concentrations in rumen fluid. Fractional absorption rate of acetic acid was decreased at both low and high initial concentrations while fractional absorption rate of propionic acid was reduced only at high initial concentrations. Accumulation of VFA within the receiving cell or limiting availabilities of ions for co- or countertransport

could be the cause of reduction in fractional absorption at high concentration levels (Bergman, 1990).

Similarly to carbohydrate metabolism, protein degradation in the rumen is the result of microbial activity and is dependent on a number of factors including protein type, ruminal dilution rate, ruminal pH, substrate being fermented and predominant species of rumen flora (Bach et al., 2005). The nitrogen source microbes use for protein synthesis consist of both dietary protein and non-protein N (NPN). True protein is incorporated into microbial protein or deaminated into ammonia N by way of degradation to peptides and amino acids (AA). This multistep process begins with microbial attachment to feed particles, which initiates peptide bonds to be enzymatically cleaved by cell-bound microbial proteases. The synergistic action of a variety of proteases is necessary for complete protein degradation due to the different bonds that exist within a single protein (Wallace et al., 1997). Free peptides and AA are absorbed rapidly by bacteria and can either be incorporated into microbial protein or further deaminated to VFA, CO_2, and ammonia (Tamminga, 1996). Amino acids will be transaminated or used directly for microbial protein synthesis if energy is available. However, AA will be deaminated and their carbon skeleton will be fermented into VFA if energy is limiting (Bach et al., 2005). Amino acids absorbed in excess will be excreted from the cytoplasm as ammonia in ruminal bacteria (Tamminga, 1996). In a general sense, efficiency of an animal's productive capacity is limited by energy intake and efficiency of energy utilization, not protein supply. However, amounts of rumen-degradable protein that exceed the amount require by microorganisms, can attribute to energy loss resulting in decreased efficiency. The excess protein is degraded to ammonia N, absorbed, and

metabolized to urea in the liver. The urea is then recycled back to the rumen for use by the microbial population or expelled from the body in the urine. Manipulation of rumen protein degradation or the efficiency of nitrogen use in the rumen is the most effective strategy to reduce nitrogen losses (Tamminga, 1996). NPN is useless in excess and can have adverse effects if it reduces feed intake or increases energy loss by the animal. The solubility and structure of a protein is a key factor in determining its susceptibility to microbial proteases. Specific peptide bonds are more resistant to ruminal degradation than others. Additionally, insoluble proteins are generally more slowly degraded (Bach et al., 2005). Several authors have indicated that protein degradation is affected by pH and type of ration, which may dictate the predominate type of microbial population present in the rumen. Cardozo et al. (2000,2002) demonstrated that protein degradation was reduced as pH decreased with both high forage and high concentrate diets. Furthermore, protein degradation was shown to be consistently lower when high-concentrate diets provided substrate to microbes vs. high-forage diets, regardless of pH. Optimal pH ranges for proteolytic enzymes range from 5.5 to 7.0 with the greatest reduction of protein degradation being at the lower end of the that range (Kopecny and Wallace, 1982). A decrease in *in vitro* pH from 6.5 to 5.7 reduced ammonia concentrations when bacteria were obtained from cattle fed 100% forage rations. In contrast, pH levels did not have an effect on reduced ammonia concentrations when bacteria were obtained from cattle fed 90% concentrate diet, indicating that forage- and concentrate-fed cattle had different populations of ammonia-producing bacteria (Lana et al., 1998).

Reduced protein degradation has been associated with pH effects as well as the type of substrate being fermented or the microbial population induced by a particular ration. This concept was demonstrated in by Devant et at. (2001) using in situ techniques to incubate soybean meal and heat-processed soybean meal in the rumen of dairy cattle fed a 60:40 ration and in beef cattle fed a 10:90 high concentrate ration. The reduced protein degradation observed with the feedlot-type ration is attributed not only to pH levels but also substrate being fermented given the fact that pH was above 6.0 with feeding of both ration types. An additional explanation for the combined effect of pH and substrate on ruminal protein degradation may be the change in microbial population. Many plant proteins are trapped in a fiber matrix that needs to be degraded before proteases can gain access for protein degradation. Protein degradation in the rumen requires a combination of enzymatic activities from various microbial species. In high-concentrate diets it is probable that even with high pH levels, starch-degrading bacteria predominate, and fiber digestion is limited by a reduction in the number of cellulolytic bacteria, reducing degradation of protein (Mould and Ørskov, 1983). A reduction in crude protein and NDF digestion was observed when pH decreased from 6.3 to 5.9. Proteolytic bacteria counts were not affected by pH, however cellulolytic bacteria counts were reduced by 50%, leading researchers to postulate that a reduction in cellulolytic bacteria as a consequence of low pH leads to the reduction in fiber degradation, reducing access of proteolytic bacteria to proteins, indirectly lessening protein degradation (Endres and Stern, 1993).

Monensin

Ionophores were approved by the Food and Drug Administration for use in ruminant animals in the mid-1970s. Ionophores are classically defined as an antibiotic because of their highly lipophilic properties, which are toxic to many bacteria and protozoa species (Pressman, 1976). The basic mode of action of an ionophore is to disrupt the movement of ions (Na^+, Ca^{2+}, K^+, H^+) across biological membranes (Schelling, 1984). Monensin is a carboxylic polyether ionophore (Haney Jr and Hoehn, 1966) that was originally developed as a coccidiostat for poultry (Richardson et al., 1976). Later work showed that monensin could also be affective in altering ruminal fermentation. Most noticeably effects have included decreased methane (Thornton and Owens, 1981; Russell and Strobel, 1988; Domescik and Martin, 1999) and ammonia (Dinius et al., 1976; Van Nevel and Demeyer, 1977b) production and changes in VFA profile; specifically increasing propionic acid production and reducing acetic acid (Richardson et al., 1976; Van Nevel and Demeyer, 1977b; Prange et al., 1978; Domescik and Martin, 1999; Quinn et al., 2009). These direct animal responses are assumed to result from system modes of action, which are a direct result of the basic modes of action. The basic mode of action of cellular ion transport (basic mode of action) results in changes that influence the rumen microbes (system mode of action) and increases animal performance (Schelling, 1984).

Ammonia can accumulate in the rumen when ruminal ammonia production exceeds the ability of ammonia-utilizing microorganisms. This excess ammonia is absorbed across the rumen wall, converted to urea by the liver then subsequently lost in urine excretion (Russell and Strobel, 1989). Ammonia production is decreased with the

addition of monensin *in vitro* and *in vivo* (Van Nevel et al., 1977 and Dennis et al., 1981, respectively). Monensin reduces ammonia-N concentration through inhibition of the hyper-ammonia-producing bacteria, a small group of ruminal bacteria that are responsible for the production of most of the ammonia (Chen and Russell, 1989). The reduction of ruminal ammonia levels are consistent with the depression of deamination and/or proteolysis (Schelling, 1984).

Depressed methane production by rumen microbes is another system mode of action of monensin. Methane production in beef cattle can be as great as 12 liters/h (Thornton and Owens, 1981) and can account for as much as 12% loss of feed energy (Russell and Strobel, 1989). The decline in methane production is often associated with changes in molar proportions of VFA. An increase in molar proportions of propionic acid with a concurrent decline in the molar proportion of acetate has been observed with feeding of ionophores (Richardson et al., 1976; Thornton et al., 1976; Van Nevel and Demeyer, 1977b; Bergen and Bates, 1984). Chen and Wolin (1979) reported that monensin selects against hydrogen-producing rumen bacteria and selects for succinate-forming bacteria as well as *Selenomonas ruminantium*, which has the ability to decarboxylate succinate to form propionate. Additionally, research by Van Nevel et al. (1977) has shown that monensin decreases the metabolism of formate to carbon dioxide and hydrogen. Both mechanisms lead to a decrease in methane formation.

Decreased acetate to propionate ratio in the rumen is a well documented resulting effect of monensin and has been viewed as a favorable change in ruminant animals (Richardson et al., 1976). Propionate production by rumen fermentation is more efficient than acetate production (Hungate, 1966; Chalupa, 1977). As discussed by Hungate and

reviewed by Chalupa, stoichiometric calculations reveal an increase in propionate production without an influence on the production of metabolic hydrogen. A greater percentage of metabolic hydrogen produced was recovered in VFA subsequently improving fermentation efficiencies (Chalupa, 1977). Furthermore, calorimetric efficiencies of utilization for maintenance of acetic acid, propionic acid and butyric acid provide evidence that propionic acid is more efficient than acetic acid by 27.3% (Blaxter, 1962). These calorimetric efficiencies were determined utilizing fasting animals continually infused with appropriate acid solution until metabolic equilibrium was reached with measurements of metabolism made throughout, including beginning and ending fasting values. A further advantage of propionate is its ability to be utilized for gluconeogenesis in addition to direct oxidation by the citric acid cycle. This can be an energetic advantage by providing more substrate for glycolysis subsequently generating more reduced coenzyme outside the mitochondrial membrane (Schelling, 1984). Evidence of this is provided by Reid (1950) wherein he concluded that intravenous injections of propionate, but not acetate, eliminate the hypoglycemic convulsions induced in sheep by insulin injections, indicating that glucose cannot be formed from acetate, but that propionic acid is a precursor of sugar. The monensin effect on volatile fatty acid production is widely accepted as one of the main system modes of action.

The effect of ruminal pH on microbial populations, fermentation products and physiological functions of the rumen make it a defining factor in the normal and stable function of the rumen. Ruminal bacteria respond to changes in diet, including increased availability of fermentable substrates, with shifts in VFA production subsequently causing shifts in pH. Changes in ruminal pH can alter the microbial population and

therefore have an influence on fermentative end products. In the case of *Streptococcus bovis*, generally considered a mixed acid fermenter utilizing glucose to produce acetate, formate and ethanol, increased substrate levels and pH lower than 5.6 can cause shifts in end-product formation to homolactic (producing only L-isomers). The increased production of lactic acid by *Strep. bovis* in a situation as described above causes ruminal pH to decline inhibiting the growth rate of most ruminal bacteria, and the acid-tolerant *Lactobacilli* (an additional lactic acid producing bacteria) become predominant (Nagaraja and Titgemeyer, 2007). Lactate depresses pH by a larger margin than similar amounts of other ruminal acids because its pK is substantially lower (3.8 vs 4.8); accumulation in the rumen will cause a considerable decrease in pH (Owens et al., 1998). Increased ruminal pH and decreased rumen lactate concentrations have been associated with monensin treatments (Nagaraja et al., 1982). The changes noted by monensin feeding occur because lactate producing bacteria, such as *Streptococcus bovis*, are monensin sensitive and the lactate utilizing bacteria, such as *Megasphaera elsdenii and Selenomonaas ruminantium*, are monensin resistant (Dennis and Nagaraja, 1981; Russell and Strobel, 1989). The differences in sensitivities of lactate-producing and –utilizing bacteria account for much of the decline in ruminal lactate concentrations and the increase in ruminal pH. Alterations in microbial populations and subsequent of declining pH levels can causes changes in the physiological function of the rumen, most notably rumen motility and absorptive function.

Direct-fed Microbials

Direct-fed microbials (DFM) are defined by the FDA as a source of viable microorganisms, with no implication regarding mode of action or benefit. This

entitlement of microbial products by the FDA is required for all products used for livestock. To date, the mechanism(s) of action facilitating performance responses to microbial products in beef cattle have not been determined. Varying types of microorganisms have been used as DFM in ruminants, including lactic acid producing bacteria such as *Lactobacillus, Streptococcus, and Enterococcus*, as well as fungal cultures including *Aspergillus oryze* and *Scaccharomyces cerevisiae* (Yoon I. K., 1998). Seo et al. (2010) also looked in the effects of lactate utilizing bacteria such as *Megasphaera elsdenii* and mixed bacterial cultures.

In a review by Krehbiel and coworkers (2003) amid 6 research trials (n=1,249 cattle) positive performance responses were observed in association with DFM-fed cattle including improvements in average daily gain, feed intake and final body weights in feedlot cattle. The effects of microbial products are not solely limited to performance responses in livestock. The concept of DFM was originally based on the potential for beneficial intestinal effects brought to light by the widespread use of probiotics in humans and livestock as a mean to fight the effects of "antibiotic diarrhea" (Yoon I. K., 1998). The effects of DFM on intestinal mucosa have been observed in pigs and chickens with indication that DFM are having a beneficial effect on the host (Baum et al., 2002; Samanya and Yamauchi, 2002). As the research on DFM continues to evolve, additional studies have provided evidence of the potential of DFM to reduce fecal shedding of *E. coli* O157:H7 (Elam et al., 2003; Peterson et al., 2007).

Microbial fermentation is required by ruminant animals in order to convert substrates unavailable for digestion by mammalian enzymes, mainly cellulose, hemicellulose and pectin, as well as for the production of microbial protein. Although

responses have been variable, direct-fed microbials have been shown to mediate biological changes in the rumen as demonstrated by changes in pH, VFA profiles, ammonia concentrations, and digestibility (Ghorbani et al., 2002; Nocek, 2002; Beauchemin et al., 2003; Nocek et al., 2003; Nocek and Kautz, 2006; Narvaez et al., 2014). The interest in the impact of DFM on ruminal fermentation is derived from its potential to prevent ruminal acidosis. Ruminal pH is a main diagnostic criterion for acidosis, a common digestive disorder in beef cattle consuming diets high in readily fermentable carbohydrates. The severity of acidosis varies from acute acidosis (accumulation of lactic acid) to subacute ruminal acidosis (total accumulation and individual proportions of VFAs; (Nagaraja and Titgemeyer, 2007). In a study conducted by Raeth-Knight et al. (2007) with Holstein cows that were fully adapted to a diet consisting of primarily corn silage, alfalfa hay, corn and soybean meal; no differences in pH were detected in cows treated with 1 x 10^9 CFU/d *Lactobacillus acidophilus* strain LA747 plus 2 x 10^9 CFU/d *Propionibacterium freudenreichii* strain PF24 or 1 x 10^9 CFU/d *Lactobacillus acidophilus* strain LA747 plus 2 x 10^9 CFU/d *Propionibacterium freudenreichii* strain PF24 plus 5 x 10^8 CFU/d *Lactobacillus acidophilus* strain LA45. More recently in an *in vitro* study utilizing inoculum from high concentrate fed steers with experimental diets top-dressed twice daily with a mixed bacterial culture, consisting primarily of *Lactobacillus acidophilus* and *Entercoccus faecium* and also including *Pediococcus acidilacticii, Lactobacillus brevis and lactobacillus plantarum,* no difference in pH levels between control and DFM treatment following a 30 h *in vitro* gas production incubation period were observed (Kenney, 2013). Observations by Nocek et al. (2002) imply that inclusion of lactate producing bacteria stimulate lactic acid utilizing

bacteria, however there may be a threshold to this stimulation. Cows fed a 70% concentrate diet treated with 10^5 CFU/d of a mixed bacterial culture *(Enterococcus faecium, Lactobacillus plantarum, and Sacchromyces cerevisiae)* had a higher mean daily pH (6.24) compared to treatments with 10^6 CFU/d (pH 5.99) and 10^7 CFU/d (pH 6.02). These data suggests that there is a level of DFM that decreases ruminal pH (10^6, 10^7), indicating acid production overwhelms acid utilization at these higher levels (Nocek, 2002). However, no differences in mean, maximum, or proportion of the day spent below a pH of 5.8 or 5.5 were detected in steers that were previously naïve to a high-concentrate diet and fed a high-concentrate, barley diet treated with 6 x 10^9 CFU *Enterococcus faecium (*a lactate producing bacteria) per day (Beauchemin et al., 2003). The lack of effect of bacterial DFM on preventing subclinical ruminal acidosis observed by Beauchemin et al. supports findings previously reported for feedlot cattle fed high-grain diets supplemented with mixed cultures of lactic acid-utilizing and lactic acid-producing bacteria (Ghorbani et al., 2002). Alterations in pH levels have been variable throughout the literature, and this variability is likely due to differences in bacterial strain, dose and substrate.

The VFA profile of the rumen is an indicator of susceptibility to subacute ruminal acidosis and can be used as a means to identify shifts in ruminal fermentation patterns. No difference in total VFA concentrations between control and *Enterococcus faecium* (6 x 10^9 CFU/d) were observes in steers fed an 87% (DM basis) steam rolled corn diet, although shifts in molar proportions of VFAs were evident. A decrease in the acetate to propionate ratio was the result of increased molar proportions of propionate at the expense of butyrate production (Beauchemin et al., 2003). In contrast, propionate

proportions remained unchanged in steers fed a nutritionally similar diet (steamed rolled barley based diet) treated with *Propionibacterium* P15 or *Propionibacterium* P15 plus *Enterococcus faecium* EF212 at a rate of 1 x 10^{10} CFU/d (Ghorbani et al., 2002). However, *Propionibacterium* P15 or *Propionibacterium* P15 plus *Enterococcus faecium* EF212 increased the molar proportion of acetate, at the expense of valerate production, above that of the control and *Enterococcus faecium* alone treated animals. Additionally, in a study by Baah et al. (2009) which assessed the effect of different levels of *Lactobacillus casei* and *Lactobacillus lactis*, researchers observed increases in the molar proportion of acetate *in vitro* and a linear increase in the acetate to propionate ratio with increasing DFM provision. An increase in the molar proportion of acetate with inoculum-provided DFM (*Lactobacillus acidophilus* and *Entercoccus faecium* and also including *Pediococcus acidilacticii*, *Lactobacillus brevis and lactobacillus plantarum*) was also observed *in vitro* by Kenney et al. (2013), along with an observed decrease in butyrate concentration. DFM are biologically active in the rumen and modulate a shift in ruminal fermentation parameters. However, these responses are inconsistent and are seemingly dependent on the strain and dosage of DFM provided.

Similarly inconsistent results have been observed for ammonia concentrations associated with differences in strain and dose of DFM provided to the animal. No effects of treatment on concentrations of NH_3 were reported in steers fed an 87% (DM basis) steam rolled corn diet treated with *Enterococcus faecium* (6 x 10^9 CFU/d) or *Enterococcus faecium* plus yeast (Beauchemin et al., 2003). Likewise, no differences in ammonia-N with DFM provision *in vitro* were observed in previously described work by Kenney et al. (2013) and Baah et al. (2009). In contrast, Ghorbani and colleagues (2002)

observed a tendency for increased NH_3 concentration in steers treated with *Propionibacterium* P15 when compared to control and *Propionibacterium* P15 plus *Enterococcus faecium* EF212. Additionally, a significantly higher NH_3-N concentration was observed in steers receiving a corn and corn DDGS-based finishing diet treated with 1 x 10^{11} CFU of *Propionibacterium acidipropionici* P169 when compared with control (P169 = 4.44mM; control = 1.05mM; (Narvaez et al., 2014).

Feeding lactic acid producing bacteria is believed to result in increased levels of lactic acid in the rumen, in turn stimulating growth of lactic acid utilizing bacteria (Nocek, 2002). Lactic acid utilizers convert lactate to propionate or succinate that can be converted to propionate. Concentrate-based diets produce proportionally more propionate than forage-based diets. The formation of propionate utilizes reducing equivalents, pyruvate is reduced to propionate, thus this pathway is considered a hydrogen utilizing pathway (Baldwin et al., 1963). Increase in propionate formation is stoichiometrically associated with a decrease in CH_4 considering H_2 as the main precursor for CH_4 production; however, to date, there have been no animal studies conducted to verify this postulation (Grainger and Beauchemin, 2011). The majority of methanogens use H_2 to reduce CO_2 in the rumen to CH_4. One of the major pathways to decrease CH_4 emissions from ruminants by using DFM is the redirection and decreased production of H_2 (Jeyanathan et al., 2014). In terms of DFM containing yeast there have been inconsistent results from studies both *in vitro* and *in vivo.* However, live yeast has shown beneficial effects on the growth and H_2-utilization of acetogenic bacteria *in vitro* (Chaucheyras et al., 1995). A recent study conducted by Alazzeh et al. (2012) provides evidence that specific strains of *Propionibacteria* mitigate methane production *in vitro*

with forage diets. *Propionibacteria* species *P. freudenreichii, P. thoenii, P. propionicus, P. jensenii* (strains T114, T159 and ATCC 4874, T83, T121 respectively) resulted in lower CH_4 production than control at all time points over a 48 h incubation period (Alazzeh et al., 2012b). These results suggest that *Propionibacteria* may have potential to be used as a DFM to mitigate CH_4 production *in vivo*, but additional research is needed.

Influence of Diet

The VFA profile of the rumen is an indication of shifts in ruminal fermentation patterns as well as the previously mentioned indicator of susceptibility to subacute ruminal acidosis. The proportions of VFA produced remains the same independent of diet, with acetate being the greatest followed by propionate and then butyrate, however the ratios of these VFAs are affected by diet. The molar proportions of VFA produced in the rumen vary depending on diet composition. In general, as the forage:concentrate ratio decreases, the acetate:propionate ratio also decreases (G.C. Fahey, 1988). Highly fermentable substrates lead to an increase in propionate production at the expense of acetate production leaving diets high in fiber with larger acetate to propionate ratios. Murphy et al. (1982) provides data to support this effect of diet composition. Generally, as cellulose and hemicellulose levels increase, relative to the amounts of soluble carbohydrate and starch levels, the acetate:propionate ratio also increases as a function of both increased acetate and decreased propionate (Murphy et al., 1982). End-product formation may vary greatly depending on diet. Fermentation of structural carbohydrates, compared to fermentation of starch, yield high amounts of acetic and low amounts of propionic acid (Dijkstra, 1994). When compared, fermentation of a substrate by animals

on roughage diets (>60% roughage) vs. fermentation of the same substrate by animals on concentrate diets (<40% roughage) generated different patterns of VFA. In terms of molar percent of total VFA concentration in rumen fluid of five Holstein-Friesian heifers, acetate proportions decreased while a large increase in propionate ensued with changes from a diet consisting of approximately 70% alfalfa hay and 30% corn meal to diets consisting solely of corn meal or steam flaked corn products (Eusebio et al., 1959). The production of both propionate and butyrate were increased as the proportion of concentrate in experimental diets increased (high forage = 699 umol, low forage = 759 umol; high forage = 456 umol, low forage = 575 umol respectively; (García-Martínez et al., 2005). Compositional make-up of the diet affects end product formation by availability of fermentative substrate as well as its effect on the viability of the microbial population (Murphy et al., 1982).

A decrease in methane emissions have been linked to various dietary components. A 27% decrease in emissions was observed in sheep when dietary oil was added to their diet (Fievez et al., 2003; Machmüller, 2006). This observed decrease is thought to be caused by the hydrogengation of fatty acids competing for substrate with and by direct inhibition of methanogens (Chaves et al., 2008). Additionally, soluble sugars have been shown to yield less methane than plant fiber (Moss et al., 1995; Hindrichsen et al., 2004). In terms of forage to concentrate (F:C) ratios , García-Martínez et al. (2005) concluded that the proportion of concentrate in a diet will significantly affect methane production. This is indicated by decreased levels of forage in the experimental diet resulting in increased methane concentration *in vitro* (high forage = 701 umol methane, low forage = 812 umol methane). On the contrary Lovett et al. (2003) reported that reducing the F:C

ratio led to a significant reduction in methane production of Charolais cross heifers fed over an 11-week period one of six diet varying in F:C ratios with and without inclusion of coconut oil. This quadratic response to a reduced F:C ratio and methane output by Lovett et al. agrees with the earlier work completed by Moss et al. (1995). Reduction of methane output resulting from a reduced F:C ratio is attributed to by shifts in ruminal fermentation patterns, in particular propionate concentrations (G.C. Fahey, 1988), as well as declines in ruminal pH (van Kessel and Russell, 1996).

The amount of non-structural carbohydrates could affect fermentation of structural carbohydrates by decreasing the pH of the rumen fluid (Sutton, 1985). Soluble carbohydrates such as starch or sugar may impede cellulose digestion by lowering pH given the fact that cellulolytic bacteria (i.e., *Ruminococcus albus, F. succinogenes, Ruminococcus flavefaciens)* are particularly sensitive to low pH (Russell and Dombrowski, 1980; G.C. Fahey, 1988). Bicarbonate, phosphate and proteins buffer the rumen, although the production of fermentation acids can sometimes exceed the buffering capacity and pH can decline to a point where rumen function and animal performance is decreased. An additional source of low rumen pH can be associated with an accumulation of lactic acid in the rumen altering the bacterial population by allowing *Streptococcus bovis* and lactobacilli to thrive (Hungate et al., 1952).

Gas Production

Chemical composition, as well as rate and extent of digestion of ruminant feed are indicators of the nutritive value of the feed. *In vitro* methods of measuring gas production have been evaluated as a means to study digestibility of feedstuffs in response to animal welfare issues, cost associated with maintaining surgically modified animal and

the limited number of samples that can be examined at one time *in vivo* (Mauricio et al., 1999). Methodologies of *in vitro* gas production estimate digestion of feeds based on measured relationships between the *in vivo* digestibility of feeds and *in vitro* gas production, in combination with the chemical composition of the feed (Menke and Steingass, 1988). An *in vitro* system was described by Menke et al. (1979) in which substrate, buffer and rumen fluid inoculum were added to a syringe and incubated at 39°C. Gas production from fermentation of the substrate was measured by reading the position of the piston at various time intervals and used to estimate digestibility and metabolizable energy. Both capacity and accuracy of this system were limited by the time required to obtain each reading and enter them manually as well as problems associated with syringe manipulation (Mauricio et al., 1999). An alternative technique was later used by Theodorou et at. (1994) where substrates incubated in sealed serum flasks caused fermentation gases to accumulate in the headspace. The accumulated gas was then measured and released using a pressure transducer assembly. Subsequent techniques were established from these proven methods of Theodorou et al. (1994) using instrumentation to obtain pressure readings with direct data capture (Mauricio et al., 1999).

Traditional *in vitro* methods measure the disappearance of the substrate; however gas production measurements focus on the appearance of both soluble and insoluble fermentation products (Pell and Schofield, 1993). VFA production is reflected in the amount of gas produced from a feed being incubated; gas arises indirectly from buffering acids generated as a result of fermentation and directly from microbial degradation of feeds (Getachew et al., 2004). Quantitative VFA production is indicated by gas

production measures. Truly digested substrates are divided among VFA, gas and microbial biomass, gas measures only account for substrate that is used for VFA and gas, therefore gas measurement can be considered only an estimate of apparent rumen digestibility, while loss of dry matter from the residue is an indication of true rumen digestibility (Blu¨mmel and Ørskov, 1993). Getachew et al. (2004) found a positive correlation between gas and VFA production. Inclusion of crude protein, non-fiber carbohydrates and fat concentrations of the substrate with gas production has been shown to improve *in vitro* gas production in a stepwise linear regression (Getachew et al., 2004). These *in vitro* findings were consistent with those of Menke and Steingass (1988) and Khazaal et al. (1995) where prediction of *in vivo* digestibility was also improved by inclusion of feed CP levels. These measurements of *in vitro* dry matter digestibility have been used widely to assess the nutritional quality of feeds due to their high correlation with *in vivo* digestibility. Traditionally conventional *in vitro* methods for determining digestibility have been compared to the ANKOM gas production system resulting in similar results (Holden, 1999).

The ANKOM system allows for high frequency detection of small changes of pressure in the headspace of the fermentation flask and is equipped with a rapid, automatic venting system that prevents incoming airflow and allows for a low pressure to be sustained. The repeatability (RT) and reproducibility (RP) of the fully automated wireless ANKOM gas production system was assessed utilizing a ring test of four different laboratories by Cornou et al. (2013). The RT of gas production measures, for this study, at 24 and 48 h (CV of 5.7 and 4.7%) were comparable with other studies using varying gas production techniques (Valentin et al., 1999; Getachew et al., 2002; Gierus et

al., 2008; Tagliapietra et al., 2011). The continuous readings provided by automated gas production systems make it possible to mathematically describe gas production curves (Cornou et al., 2013). Standard reporting of gas production requires the change in pressure (in units of psi), reported by the ANKOM system, to be converted to gas volume (mL) at standard temperature and pressure (STP) and can be calculated according to the following equation:

$$V2 = \frac{p1 \times V1}{p2} \times \frac{T2}{T1}$$

Where, V2=volume at STP; p1=pressure transducer reading (psi); V1=volume of headspace p2=standard pressure (14.7 psi); T2=standard temperature (273K) and T1=temperature at measurement conditions (312K).

CHAPTER 2: Effect of direct-fed microbials and monensin on *in vitro* fermentation of a high-forage substrate

INTRODUCTION

Inclusion of DFM in both receiving and finishing diets in beef cattle has been shown to improve intake, as well as rate and efficiency of gain (Swinney-Floyd et al., 1999; Elam et al., 2003; Krehbiel et al., 2003). Although the mechanism(s) responsible for these improvements in performance have not been completely elucidated, there is evidence that DFM alter rumen fermentation characteristics (i.e. VFA concentrations, methane production, microbial population) (Ghorbani et al., 2002; Nocek, 2002; Beauchemin et al., 2003; Nocek et al., 2003). Most commonly, a mixed bacterial culture of lactic acid producing and utilizing, gram positive bacteria are used as DFM in ruminants (Ghorbani et al., 2002). Lactic acid producing bacteria such as *Lactobacillus* and *Streptococcus* (Yoon I. K., 1998), and lactate utilizing bacteria such as *Megasphaera elsdenii* (Seo et al., 2010) have been investigated individually as well as in combination to identify their specific beneficial effects on the rumen environment. Recent research from our laboratory has shown that mixed bacterial cultures consisting primarily of *Lactobacillus acidophilus* and *Enterococcus faecium* when used in combination with a high concentrate ration, decreases *in vitro* gas production as well as total VFA concentration (Kenney, 2013). Additionally, *in vivo,* this mixed culture DFM decreased total VFA concentration and increased molar proportions of acetate leading to an increase in pH (Kenney, 2013). However, little is known about this mixed culture DFM (consisting primarily of *Lactobacillus acidophilus* and *Enterococcus faecium*) in combination with a forage-based diet.

Microbial populations are altered by changes in dietary composition leading to higher counts of fibrolytic bacteria with increased forage levels (Tajima et al., 2001). *Fibrobacter succinogenes* (gram -), *Ruminococcus flavefaciens* (gram +) and *Ruminococcus albus* (gram +) are considered representative fibrolytic bacteria of the rumen (Forsberg et al., 1997; Koike et al., 2003). These shifts in microbial population have the potential to alter VFA production and subsequently pH levels in the rumen.

Ionophores are a feed-grade antibiotic used in cattle diets to enhance feed efficiency and body weight gain. Ionophores selectively inhibit the metabolism of gram-positive bacteria, which lack a protective outer membrane, and protozoa in the rumen. Monensin is a carboxylic polyether ionophore (Haney Jr and Hoehn, 1966) that has been shown to alter ruminal fermentation by selecting against hydrogen-producing bacteria reducing the substrate for methane production (Chen and Wolin, 1979). Moreover, monensin decreases ammonia production through inhibition of the hyper-ammonia-producing bacteria, a small group of ruminal bacteria that are responsible for the production of most of the ammonia (Chen and Russell, 1989). Additionally, monensin elicits changes in VFA profile, specifically increasing propionic acid production and reducing acetic acid (Richardson et al., 1976; Van Nevel and Demeyer, 1977b; Prange et al., 1978).

Monensin is widely used in receiving cattle rations as well as in some forage-based production systems (Raun et al., 1976; Martinez et al., 2009). The biological benefits of DFM have led to an increase in use. However, little is known about the effects of combined DFM and monensin treatments. Monensin's ability to selectively inhibit gram-positive bacteria in combination with the gram-positive nature of DFM

suggests the possibility of a direct interaction between there dietary addition. However, other possibilities for interaction between the two exist, given that both have potential to modify the microbial ecosystem in a variety of ways. The objective of this study was to determine the effect of a mixed bacterial culture, previously used by our lab, consisting primarily of *Lactobacillus acidophilus* and *Enterococcus faecium*, on *in vitro* fermentation and methane production from a forage substrate, with and without the addition of monensin.

MATERIALS AND METHODS

All procedures were approved by the University of Kentucky Institutional Animal Care and Use Committee.

Donor animals and diet. Four ruminally cannulated steers (396-440 kg) were housed indoors in individual pens (2.4 x 2.6 m) and fed an 80:20 forage: concentrate diet (Table 2.1) at 1.75 x NEm . The steers were adapted to the experimental diet over a 15 d span prior to sampling of rumen contents and were fed twice daily (8AM and 5PM) during the experimental period.

Treatments. All treatments were applied *in vitro* and were arranges as a 2 x 2 factorial. Treatments included 2 levels of DFM (0 and 50,000 cfu) and 2 levels of monensin (0 and 5 ppm). The DFM contained a mixed culture of bacteria consisting of primarily *Lactobacillus acidophilus* and *Enterococcus faecium* and also included *Pediococcus acidilaticii, Lactobacillus brevis, and Lactobacillus plantarum* (10-G, Vit-E-Men Co., Norforlk, NE, USA; precise proportions are proprietary). Media DFM dosage (cfu/unit of dry matter) was based on previous *in vivo* studies with the same DFM that have shown enhanced animal performance and alterations in rumen fermentation

(Kenney, 2013). Direct-fed Microbial treatments were prepared by dissolving 0.05 g DFM plus carrier or carrier (lactose) into 100 mL distilled water and 1.0 mL of each solution was added to the appropriate vessels. Similarly, monensin treatments were prepared by dissolving 473.2 mg monensin sodium salt into 47.32 mL 100% ethanol and 50 uL of the monensin containing solution or ethanol was added to the appropriate vessels.

In vitro procedures. Ruminal contents were collected from the ventral rumen of each steer approximately 1 h after AM feeding. Contents from each of the four steers were stored in separate insulated containers for transport into the lab. Prior to processing, the entire contents from each individual animal were combined into a large insulated container. The combined ruminal contents were strained through 4 layers of cheesecloth and the resulting fluid, along with a grab sample of whole contents, were processed using an immersion blender for two minutes under a CO_2 headspace. The blended contents were strained a second time through 4 layers of cheesecloth to form the inoculum source for the experiment. *In vitro* gas production was determined on 4 separate days, with each run consisting of 4 vessels/treatment (n = 12/treatment). Buffer solution, micro- and macro-mineral solutions, and reducing solutions were prepared as described previously (Goering and Van Soest, 1970). A combination of prepared solutions (1475 mL) and 350 mL of rumen inoculum (media solution) were maintained in a 39°C water bath under a CO_2 environment until added to the 250 mL fermentation vessels. Fermentation vessels were supplied with common substrate (400 mg of donor diet (Table 2.1); the diet was ground with a Wiley Mill to pass through a 1-mm screen. Each fermentation vessel also received 2 mL of H_2O (to prevent suspension of feed particles outside of liquid solution),

100 mL of media solution and appropriate amounts of each treatment solution (1mL DFM solution or lactose carrier, and 50 uL monensin solution or ethanol). Subsequently, vessels were gassed with CO_2 for 30 seconds and then fitted with remote automatic pressure transducers (AnkomRF Wireless Gas Production System, Ankom Technology, Macedon, NY). Vessels were incubated in a water bath at 39˚C for 30 hours, to ensure plateau gas pressure was reached, and gas pressure was measured at 5-minute intervals. At the completion of the 30-h fermentation, vessels were placed into an ice bath to cease fermentation and gas samples were drawn into10mL red-topped serum vacutainer tubes for methane analysis. After gas sampling, flasks were opened, pH was immediately determined using a portable pH meter (Acorn pH 6 Meter, Oakton Instruments, Vernon Hills, IL, USA), and samples of the culture broth were collected. A 5 mL aliquot of the sample was added to 15mL Nunc screw-cap centrifuge tubes containing 0.5 mL of metaphosphoric acid (25 g/100 mL) and 0.5 mL of volatile fatty acid (VFA) internal standard (1 g/100 mL 2-ethlybutyrate) and frozen for later VFA analysis. Additionally, a 100 uL sample was combined with 3.9 mL phosphoric acid (25 mM H_3PO_4) and frozen for ammonia analysis.

Sample analysis. VFA concentrations of the culture broth were determined by gas chromatography (6890 Hewlett-Packard, Avondale, PA), fitted with a Supelco 25326 Nukol fused silica capillary column (15m x 0.53mm x 0.05um film thickness; Sigma/Supelco, Bellefonte, PA) following previously described procedures (Erwin et al., 1961; Ottenstein and Bartley, 1971). Ammonia-N concentration was determined using a photometric test with enzymatic assay through Konelab analysis (Model 20XTi, Thermo Fisher Scientific, Waltham, MA) following procedures previously described by Kun and

Kearny (1974). Additionally, gas samples were analyzed for methane concentration by gas chromatography (6890 Hewlett-Packard, Avondale, PA), fitted with a Supelco stainless steel 40/60 carboxen 1000 packed column (5ft x 1/8in x 2.1mm). Column head pressure was set at 10psi and oven temperature set point was 125°C (maximum 190°C) (Xu et al., 2010).

Calculations. Cumulative gas pressure readings were converted to gas volumes at standard temperature and pressure using the ideal gas law. Converted gas volumes of individual modules were quantified using the best fit model from the evaluation of ten gas production models evaluated by Pitt et al. (1999), in addition to an exponential model without a lag period ($V = VF(1 - e^{-kt})$ where V = gas volume at time (t) and VF = gas volume at plateau (Wang et al., 2011). All model parameters and curve fit statistics were generated using nonlinear least squares methods in MATLAB (Version R2013a, Mathworks, Natick, MA). The best fit curves were determined as those with the lowest RMSE values. The exponential model without lag, described above, was determined to be the best-fit model for gas production data and was used to calculate rate and total production of gas.

Statistical analysis. Exponential model parameters of *in vitro* gas production (gas volume at plateau and rate of gas production) and fermentation end products (VFA, ammonia and methane) were analyzed using a model appropriate for a randomized complete block design, with blocks representing separate runs of the *in vitro* procedures conducted on each of 4 separate days. The data were analyzed using the GLM procedure in SAS (SAS Inst. Inc., Cary, NC). The model statement included main effects (DFM and Monensin) and their interactions, as well as block. Multiple replications of treatments

within each run permitted initial analysis to evaluate run x treatment interactions. This preliminary analysis revealed run x treatment interactions ($P < 0.01$) for either DFM and/or MON treatments with all fermentative end products, rate of gas production, and end point pH values which highlighted a single run containing spurious values. This run was removed from subsequent analysis. No other run x treatment interactions were detected ($P > 0.10$) and thus, the interaction term was removed from final statistical analysis for all response variables.

RESULTS

The addition of DFM did not affect ($P > 0.10$) rate or total gas production (Table 3.2). In contrast, MON decreased ($P < 0.01$) both total gas production as well as rate of gas production (Table 2.2).

Monensin decreased ($P < 0.01$) total VFA concentration while DFM tended to increase ($P = 0.06$) total VFA concentration (Table 2.3). However, there was a tendency ($P = 0.07$) for a DFM x MON interaction for total VFA concentration where DFM increased total VFA concentration in the absence but not in the presence of MON. There were no other DFM x MON interactions ($P > 0.11$) for any of the other variables.

The provision of MON altered the concentration of all VFAs (Table 2.3). Monensin decreased ($P < 0.01$) acetate and butyrate concentrations, and increased ($P < 0.01$) the concentrations of propionate, isobutyrate, isovalerate and valerate. In contrast to MON, there was no effect of DFM on VFA concentrations except for a tendency to decrease ($P = 0.08$) isovalerate concentration. Monensin decreased ($P < 0.01$) the molar proportions of acetate and butyrate and increased ($P < 0.01$) molar proportions of propionate, valerate, isobutyrate and isovalerate. Similar to the effect of DFM on VFA

concentrations, the effect of DFM was absent for molar proportion of VFAs with the exception that DFM slightly decreased (P = 0.08) the molar proportion of isovalerate. Additionally, MON decreased (P < 0.01) the acetate to propionate ratio (4.33 vs 3.15), while DFM had no effect (P > 0.10).

Ammonia-N was decreased (P < 0.01) by MON, 21.2 mmol/L for control and 19.2 mmol/L for MON. In contrast, ammonia-N was not impacted (P = 0.57) by DFM. Direct-fed microbial and MON increased (P < 0.01) end point pH similarly resulting in an additive effect when the two treatments were combined (Table 2.3). Monensin decreased (P < 0.01) total amount and percent of methane produced, (23.22 vs. 14.47 mL, 23.85% vs. 18.63%) while DFM had no effect (P = 0.61;Table 2.2).

DISCUSSION

In vitro techniques attempt to simulate the rumen environment to allow for characterization of treatment-mediated changes within the rumen. Total gas produced is generally increased as substrate disappearance increases indicating a positive relationship between extent of feedstuff degradation and *in vitro* gas accumulation (Theodorou et al., 1994). In the current experiment, DFM treatment, with a mixed bacterial culture of lactate producing DFM primarily consisting of *Lactobacillus acidophilus* and *Enterococcus faecium*, resulted in no significant changes in total gas production or rate of gas production. Monensin decreased both rate and extent of gas production. Baah et al. (2009) observed a linear decrease in total gas production after 12 h of fermentation, relative to control, with provision of increasing levels of lactate producing DFM (i.e. *Lactobacillus casei* and *Lactobacillus* lactis) in a 60:40 forage:concentrate barley-silage-

based diet. However, similar to the current study, no differences in total gas production were observed for 6, 24, and 48 h fermentations. While limited data is available on gas production measures with the inclusion of DFM in forage-based diets, more is known about the effect of DFM in vitro with concentrate diets. Previous research from our lab using the same inoculum DFM treatment, with high concentrate substrate, resulted in a decrease in total gas production, indicating a decrease in substrate degradation and reduced rumen fermentation (Kenney, 2013). Additionally, Baah et al. (2009) reported a linear decrease in total gas production with inclusion of previously mentioned DFM in a 10:90 forage:concentrate barley-grain-based diet. In concert with the current findings, this suggests that DFM, particularly lactate producing bacteria, effects on gas production is dependent, at least in part, on time of incubation and substrate fermented.

Dissimilar to DFM, there was a positive relationship between gas production and total VFA concentrations with inclusion of MON treatments, indicating a decrease in dry matter disappearance. Although limited data is available on the effect of monensin on *in vitro* gas production with use of forage-based inoculum, more is known about monensin effects on concentrate diets. Quinn et al. (2009) reported average total gas production was 5.9% less than control with monensin treatment on *in vitro* fermentation of steam-flaked corn and cottonseed meal substrate. These results were similar to those reported by Callaway and Martin (1996), where *in vitro* culture with added monensin had less total gas production than those not receiving monensin.

Although DFM did not mediate a change in gas production parameters, it did tend to increase total VFA production in the absences of MON without altering VFA proportions. Baah et al. (2009) observed a linear increase in total VFA production, as

compared to control, with provision of increasing levels of *Lactobacillus casei* and *Lactobacillus lactis* after 6 and 12 h of *in vitro* fermentations; however, a linear decrease was observed at 24 h fermentation. In the same study, linear increase in acetate to propionate ratio was observed with increasing DFM provision after 12, 24 and 48 h of fermentation. It is unknown whether similar results would have been observed in the present study given analyses were only obtained on 30 h data. In contrast, Raeth-Knight et al. (2007) observed no difference in total VFA concentrations or molar proportions of individual VFA, *in vivo*, among mixed lactate producing and utilizing DFM treatments (DFM1 *Lactobacillus acidophilus* strain LA747 and *Propionibacterium freudenreichii*; DFM2 *Lactobacillus acidophilus* strains LA747and LA45 and *Propionibacterium freudenreichii*) and control treatments.

In contrast to DFM, monensin decreased total VFA concentration and shifted the molar proportion of acetate and propionate such that there was a decrease in the acetate-propionate ratio. Additionally, monensin decreased butyrate levels and increased isobutyrate, valerate and isovalerate levels. Treatment effects were absent for total VFA concentrations in previous research with inclusion of varying levels of monensin in forage based diets (Potter et al., 1976; Ramanzin et al., 1997; Packer et al., 2011). Similar to the findings of the present study, Richardson et al. (1976) and Ramanzin et al. (1997) observed decreased proportions of acetic and butyric acids with concurrent increased proportion of propionic acid with inclusion of monensin *in vitro* and *in vivo* (respectively).

Both DFM and MON are commonly incorporated into cattle diets. However, there is a paucity of information in the literature concerning the potential interaction

between DFM and MON. In addition to the main effect of both additives there was a tendency for DFM x MON interaction on total VFA concentration, while no other interactions were observed. DFM tended to increase total VFA concentration in the absence but not in the presence of MON. The ability of DFM to alter total VFA concentrations in the absence but the not presence of MON, suggests that monensin may be impacting the ruminal effects of the DFM. The basic mode of action of an ionophore is to disrupt the movement of ions (Na^+, Ca^{2+}, K^+, H^+) across biological membranes (Schelling, 1984). Ionophores selectively inhibit the metabolism of gram-positive bacteria, which lack a protective outer membrane, and protozoa in the rumen. Monensin's ability to inhibit lactate-producing rumen bacteria, *S. bovis* and *Lactobacillus* species, was observed by Dennis et al. (1981) in sensitivity and growth rate trials. Similar results were reported in which monensin significantly decreased the lactobacilli and enterococci counts in *in vitro* incubations of the crop contents of chickens not previously exposed to monensin treatments (Rada and Marounek, 1996). This research suggests that monensin may have an adverse effect on the DFM treatments in the present study given the DFM primarily consist of *Lactobacillus acidophilus* and *Enterococcus faecium.* Additionally, Van Nevel et al. (1977) reported efficiency for both total and net microbial growth were significantly depressed by addition of monensin in an *in vitro* study as determined by measuring the incorporation of ^{32}P-labeled phosphate in microbial material, which is in agreement with other data obtained *in vitro* as well as *in vivo* (Van Nevel and Demeyer, 1977a). This would suggest that monensin could either directly inhibit the DFM bacteria and/or other bacteria that may be positively influenced by the DFM but inhibited by monensin.

In the present study, monensin decreased total VFA concentration and increased end point pH levels of culture broth. These observations are inconsistent with previous *in vivo* studies in cattle consuming high forage diets which have shown the inclusion of monensin to have no effect on total VFA concentration or rumen pH levels (Dinius et al., 1976; Packer et al., 2011). The reason for this discrepancy is unclear. In contrast, inclusion of DFM treatment resulted in increased pH of the culture broth combined with increased total VFA concentrations, particularly in the absence of monensin. Nonetheless, it has been well demonstrated across a variety of diets that there is an inverse relationship between rumen pH and total VFA concentrations (Phillipson, 1942; Briggs et al., 1957). Other research reported no difference in rumen pH with inclusion of various strains and combinations of *Lactobacillus acidophilus and Propionibacterium* in forage-based diets (Raeth-Knight et al., 2007; Sanchez et al., 2014). Given the scope of this experiment it is difficult to ascribe a mechanism to account for the currently observed increase in both pH and total VFA concentrations. Inclusion of the current DFM has resulted in increased levels of total VFA in combination with decreased pH, *in vitro*, with high concentrate substrate (Kenney, 2013). This is an indication that DFM-modulated changes could be dependent on diet.

Monensin reduces ammonia-N concentration through inhibition of the hyper-ammonia producing bacteria; a small group of ruminal bacteria that are responsible for the production of most of the ammonia (Chen and Russell, 1989). In the present study monensin decreased levels of ammonia when used *in vitro* with high forage substrate. Similar results were observed *in vitro* using a timothy hay substrate with the inclusion of monensin, where monensin caused a significant decrease in ammonia accumulation

(Russell and Strobel, 1988). Additionally, monensin appeared to decrease ammonia concentrations levels *in vivo* with a 90% orchardgrass diet although the differences among treatments were not significant (Dinius et al., 1976). Ammonia-N did not differ with provision of DFM, which is in agreement with previous work that has found no differences in ammonia concentrations or microbial N with DFM provision *in vitro* and *in vivo* (Raeth-Knight et al., 2007; Baah et al., 2009).

Monensin decreased methane concentrations with inclusion *in vitro* with a high forage substrate. Similar results have been observed *in vitro* using a timothy hay substrate with the inclusion of monensin, where monensin caused a significant decrease in methane production (Russell and Strobel, 1988). Additionally, monensin decreased methane production *in vivo* when included in forage-based diets consisting of corn silage/haylage and barley silage (McGinn et al., 2004; Odongo et al., 2007b). Benefits of feeding monensin include a shift in the acetate to-propionate ratio toward more propionate and an associated decrease in methanogenesis (Russell and Houlihan, 2003). Consistent with these findings, monensin decreased methane and increased propionate in the present study.

There was no significant effect of DFM on methane production. Increased levels of acetate are indicative of increased methane production (as previously mentioned); consistent with our results neither end product was affected by DFM provision. Limited data is available with direct measure of methane as affected by inclusion of DFM. However, it has been suggested that some DFM may redirect H_2 reducing its availability for use in methane production (Jeyanathan et al., 2014). In terms of DFM containing yeast, there have been inconsistent results from studies both *in vitro* and *in vivo,* however,

live yeast have shown beneficial effects on the growth and H_2-utilization of acetogenic bacteria *in vitro* (Chaucheyras et al., 1995). Acetogenic bacteria reduce carbon dioxide to acetate and in this reduction they are competing with methanogens for hydrogen subsequently reducing CH_4 production (Alazzeh et al., 2012a). An additional strategy to mitigate CH_4 in ruminants is to increase competition for hydrogen by producing more propionate in the rumen (Guan et al., 2006; Odongo et al., 2007a). A recent study conducted by Alazzeh et al. (2012) provides evidence that specific strains of *Propionibacteria,* which produce propionate as an end product of fermentation, could help mitigate methane production *in vitro* with forage diets.

Table 2.1 Dry matter and nutrient composition of high forage *in vitro* substrate and donor diet

Ingredient	%, DM basis
Alfalfa, cube	80.00
Cracked Corn	18.95
Soybean Meal	0.50
Fat	0.05
Urea	0.16
Limestone	0.28
Trace Mineral-Salt	0.08
Vitamin A,D & E Premix	0.0006
Nutrient, DM basis[a]	80%Forage:20%Concentrate
Crude Protein, %	13.03
Acid Detergent Fiber, %	26.11
Neutral Detergent Fiber, %	34.65
NFC, %	13.60
Calcium, %	1.11
Phosphorus, %	0.17
Magnesium, %	0.15
Potassium, %	0.97
Sodium, %	0.08
Sulfur, %	0.15
Iron, ppm	125.96
Zinc, ppm	21.20
Copper, ppm	7.30
Manganese, ppm	28.02
Molybdenum, ppm	1.06
NE_m, Mcal/kg	1.03
NE_g, Mcal /kg	0.57

[a] Based on analyses by Dairy One, Ithaca, NY.

Table 2.2 Effect of direct-fed microbial and monensin on rate and extent of gas production and total methane production

	Treatment					P-Value		
	(-) DFM		(+) DFM					
	(-) MON	(+) MON	(-) MON	(+) MON	SEM	MON	DFM	DFM*MON
Rate, hr^{-1}	0.120	0.107	0.121	0.106	0.001	<0.01	0.93	0.15
Plateau, mL	100.82	82.85	101.53	82.65	0.757	<0.01	0.74	0.55
Methane, %	23.85	18.63	23.67	18.21	0.249	<0.01	0.23	0.63
Methane, mL	23.22	14.47	23.26	14.15	0.274	<0.01	0.61	0.52

Table 2.3 The effect of direct-fed microbial and monensin on *in vitro* fermentative end products of a high forage diet

	Treatment					P-Value		
	(-) DFM		(+) DFM					
	(-) MON	(+) MON	(-) MON	(+) MON	SEM[a]	MON	DFM	DFM*MON
Total VFA, mM	71.42	65.81	73.20	65.87	0.47	<0.01	0.06	0.07
Acetate	65.76	61.18	66.02	61.18	0.11	<0.01	0.25	0.26
Propionate	15.28	19.40	15.18	19.50	0.09	<0.01	1.00	0.32
Isobutyrate	1.87	1.92	1.84	1.91	0.02	<0.01	0.35	0.57
Butyrate	10.20	9.74	10.16	9.69	0.06	<0.01	0.43	0.90
Isovalerate	3.55	3.69	3.50	3.68	0.02	<0.01	0.08	0.43
Valerate	3.35	4.06	3.30	4.05	0.02	<0.01	0.18	0.53
Molar Proportion moles/100moles								
Acetate	65.80	61.20	66.00	61.20	0.11	<0.01	0.25	0.26
Propionate	15.30	19.40	15.20	19.50	0.09	<0.01	1.00	0.32
Isobutyrate	1.90	1.90	1.80	1.90	0.02	<0.01	0.35	0.57
Butyrate	10.20	9.70	10.20	9.70	0.06	<0.01	0.43	0.90
Isovalerate	3.50	3.70	3.50	3.70	0.02	<0.01	0.08	0.43
Valerate	3.30	4.10	3.30	4.00	0.03	<0.01	0.18	0.53
Acetate:Propionate	4.31	3.16	4.35	3.15	0.02	<0.01	0.43	0.11
NH_3,mM	20.90	19.60	21.40	18.70	0.4	<0.01	0.57	0.11
pH	6.49	6.57	6.57	6.67	0.02	<0.01	<0.01	0.61

CHAPTER 3: Effect of direct-fed microbials and monensin on *in vitro* fermentation of a high-concentrate substrate

INTRODUCTION

Inclusion of DFM in both receiving and finishing beef cattle diets has been shown to improve intake, and rate and efficiency of gain (Swinney-Floyd et al., 1999; Elam et al., 2003; Krehbiel et al., 2003). Feeding bacterial DFM to feedlot cattle results in 2.5 to 5% increase in daily gain and approximately a 2% increase in feed efficiency as reported by Krehbiel et al. (2003) in a review of literature. Although mechanism(s) responsible for these improvements in performance have not been completely elucidated, there is evidence that DFM alter rumen fermentation characteristics (i.e. VFA concentrations, methane production, microbial population) (Ghorbani et al., 2002; Nocek, 2002; Beauchemin et al., 2003; Nocek et al., 2003). Varying types of microorganisms have been used as DFM in ruminants, including lactic acid producing bacteria such as *Lactobacillus* and *Streptococcus* (Yoon I. K., 1998), as well as lactate utilizing bacteria such as *Megasphaera elsdenii* (Seo et al., 2010). The ability of DFM to alter ruminal fermentation with shifts in rumen pH and VFA profiles has led to interest in the application of DFM as a preventative tool against ruminal acidosis. Hauffman et al. (1992) reported that *Lactobacillus acidophilus* had reduced the time ruminal pH was below 6.0 in steers. Nocek et al. (2000) reported daily low pH was higher, and area under pH 5.5 was lower for dairy cows receiving *Enterococcus* and *Lactobacillus*. Recent research from our laboratory has shown that mixed bacterial cultures consisting primarily of *Lactobacillus acidophilus* and *Enterococcus faecium,* when used in

combination with a high concentrate ration, decreased *in vitro* gas production as well as total VFA concentration (Kenney, 2013). Additionally, *in vivo,* this mixed culture DFM decreased total VFA concentration, leading to an increase in pH, and increased molar proportions of acetate (Kenney, 2013).

Ionophores are feed additives used in cattle diets to increase feed efficiency and body weight gain. Ionophores selectively inhibit the metabolism of gram-positive bacteria, which lack a protective outer membrane, and protozoa in the rumen. Monensin is a carboxylic polyether ionophore (Haney Jr and Hoehn, 1966) that has been shown to alter ruminal fermentation by decreasing methane by direct inhibition of methanogens (Chaves et al., 2008) and ammonia production through inhibition of the hyper-ammonia-producing bacteria, a small group of ruminal bacteria that are responsible for the production of most of the ammonia (Chen and Russell, 1989). Decreased acetate to propionate ratio in the rumen is a well documented resulting effect of monensin and has been viewed as a favorable change in ruminant animals (Richardson et al., 1976). In general, as the forage:concentrate ratio decreases, the acetate:propionate ratio also decreases (G.C. Fahey, 1988). Propionate production by rumen fermentation is more efficient than acetate production (Hungate, 1966; Chalupa, 1977) and can be an energetic advantage by providing more substrate for glycolysis (Schelling, 1984). Inclusion of monensin in high concentrate diets has shown no significant effect on total VFA concentration (Raun et al., 1976; Guan et al., 2006; Ellis et al., 2012).

Since monensin's approval in feedlot diets (1975) it has gained acceptance by the cattle feeding industry for its ability to improve growth efficiencies when included in a variety of diets. Inclusion of monensin with high-concentrate feedlot diets has resulted in

reduced methane losses, fed intake, lactic acid production and is indicated for reduction of feedlot bloat (Goodrich et al., 1984). Additionally, the biological benefits of DFM have led to an increase in use although little is known about the effects of combined DFM and monensin treatments. Commonly used DFM in commercial operations include lactate producing, gram-positive bacteria. Monensin's ability to selectively inhibit gram-positive bacteria in combination with the gram-positive nature of DFM creates the potential for direct interactions between these two feed additives. The objective of this study was to determine the effect of a mixed bacterial culture, consisting primarily of *Lactobacillus acidophilus* and *Enterococcus faecium*, on *in vitro* fermentation parameters of a concentrate-based substrate with and without the addition of monensin.

MATERIALS AND METHODS

All procedures were approved by the University of Kentucky Institutional Animal Care and Use Committee.

Donor animals and diet. Four ruminally cannulated steers (396-440 kg) were housed indoors in individual pens (3.0 x 3.7 m) and fed an 20:80 forage: concentrate diet (Table 3.1) at 1.75 x NEm . The steers were adapted to the experimental diet over a 15 d span prior to sampling of rumen contents and were fed twice daily (8AM and 5PM) during the experimental period.

Treatments. All treatments were applied *in vitro*, and were arranged in a 2 x 2 factorial structure. Treatments included 2 levels of DFM (0 and 50,000 cfu) and 2 levels of monensin (0 and 5 ppm). The DFM was a mixed bacteria culture consisting primarily of *Lactobacillus acidophilus* and *Enterococcus faecium* and also included *Pediococcus*

acidilaticii, Lactobacillus brevis, and *Lactobacillus plantarum* (10-G, Vit-E-Men Co., Norforlk, NE, USA; precise proportions are proprietary). Media DFM dosage (cfu/unit of dry matter) was based on previous *in vivo* studies with this DFM that have shown enhanced animal performance and alterations in rumen fermentation (Kenney, 2013). Direct-fed microbial treatments were prepared by dissolving 0.05 g DFM plus carrier or carrier (lactose) into 100mL distilled water and 1.0 mL of each solution was added to the appropriate vessels. Similarly, monensin treatments were prepared by dissolving 473.2 mg monensin sodium salt into 47.32 mL 100% ethanol and 50 uL of the monensin containing solution or ethanol was added to the appropriate vessels.

In vitro procedures. Ruminal contents were collected from the ventral rumen of each steer approximately 1 h after AM feeding. Contents from each of the four steers were stored in separate insulated containers for transport into the lab. Prior to processing the entire contents from each individual animal were combined into a large insulated container. The combined ruminal contents were strained through 4 layers of cheesecloth and the resulting fluid, along with a grab sample of whole contents, were processed using an immersion blender for two minutes under a CO_2 headspace. The blended contents were strained a second time through 4 layers of cheesecloth to form the inoculum source for the experiment.

In vitro gas production was determined on 4 separate days, with each run consisting of 4 vessels/treatment (n = 12/treatment). Buffer solution, micro- and macro-mineral solutions, and reducing solutions were prepared as described previously (Goering and Van Soest, 1970). A combination of prepared solutions (1475 mL) and 350 mL of rumen inoculum (media solution) were maintained in a 39°C water bath under a CO_2

environment until added to the 250 mL fermentation vessels. Fermentation vessels were supplied with common substrate (400 mg of donor diet (Table 3.1); the diet was ground with a Wiley Mill to pass through a 1-mm screen. Each fermentation vessel also received 2 mL of H_2O (to prevent suspension of feed particles outside of liquid solution), 100 mL of media solution and appropriate amounts of each treatment solution (1mL DFM solution or lactose carrier, and 50 uL monensin solution or ethanol). Subsequently, vessels were gassed with CO_2 for 30 seconds and then fitted with remote automatic pressure transducers (AnkomRF Wireless Gas Production System, Ankom Technology, Macedon, NY). Vessels were incubated in a water bath at 39˚C for 30 hours to ensure plateau gas pressure was reached, and gas pressure was measured at 5-minute intervals. At the completion of the 30-h fermentation, vessels were placed into an ice bath to cease fermentation. Flasks were opened, pH was immediately determined using a portable pH meter (Acorn pH 6 Meter, Oakton Instruments, Vernon Hills, IL, USA), and samples of the culture broth were collected. A 5 mL aliquot of the sample was added to 15mL Nunc screw-cap centrifuge tubes containing 0.5 mL of metaphosphoric acid (25 g/100 ml) and 0.5 mL of volatile fatty acid (VFA) internal standard (1 g/100 mL 2-ethlybutyrate) and frozen for later VFA analysis. Additionally, a 100 uL sample of culture broth was combined with 3.9 mL phosphoric acid (25 mM H_3PO_4) and frozen for ammonia analysis.

Calculations. Cumulative gas pressure readings were converted to gas volumes at standard temperature and pressure using the ideal gas law. Converted gas volumes of individual modules were quantified using the best fit model from the evaluation of ten gas production models evaluated by Pitt et al. (1999), in addition to an exponential model

without a lag period ($V = VF(1 – e^{-kt})$ where V = gas volume at time (t) and VF = gas volume at plateau (Wang et al., 2011). All model parameters and curve fit statistics were generated using nonlinear least squares methods in MATLAB (Version R2013a, Mathworks, Natick, MA). The best-fit curves were determined as those with the lowest RMSE values. The exponential model without lag, described above, was determined to be the best-fit model for gas production data and was used to calculate rate and total production of gas.

Statistical analysis. Exponential model parameters of *in vitro* gas production (gas volume at plateau and rate of gas production) and fermentation end products (VFA, ammonia and methane) were analyzed a model appropriate for a randomized complete block design, with blocks representing separate runs of the *in vitro* procedures conducted on each of 4 separate days. Identification of outliers were determined, using SAS JMP ver. 10.0.0, after run x treatment interactions were found indicating single parameter differences. For each response variable, residuals were determined after fitting the appropriate model. Residual values for individual replicates that exceeded the 3rd quartile + 1.5 x (interquartile range) or less than the 1st quartile – 1.5 x (interquartile range) were considered outliers and excluded from calculations. The data were analyzed using the GLM procedures in SAS (SAS Inst. Inc., Cary, NC). The model statement included direct-fed microbial treatment, monensin treatment and their interactions, as well as block. As in Expt. 1, preliminary statistical analysis included the run x treatment interaction in the model. No such interactions were detected ($P > 0.10$) and therefore this interaction term was removed from the model for final analysis.

RESULTS

In vitro gas production exhibited a DFM x MON interaction ($P < 0.01$; Table 4.2). Addition of DFM increased ($P < 0.01$) gas production, although the observed increase was partially, but not totally, abated by the presence of MON. Addition of MON alone however, decreased ($P < 0.01$) gas production in the presence of DFM but had no effect ($P = 0.60$) in the absence of DFM. A similar DFM x MON interaction tended ($P = 0.07$) to occur for rate of gas production. In this case rate was increased ($P < 0.01$) by DFM in the absence but not the presence of MON.

Direct-fed microbial x monensin interactions were present ($P \leq 0.05$) for total VFA concentrations as well as for individual VFA concentrations (Table 4.3). Monensin increased ($P < 0.01$) total VFA and valerate concentrations in the absence of DFM however, had no effect ($P > 0.10$) in the presence of DFM. Additionally, DFM increased ($P < 0.05$) total VFA and valerate concentrations in the absence of MON but decreased ($P < 0.05$) these concentrations in the presence of MON. Monensin increased ($P <0.01$) acetate concentration in the absence of DFM while having no effect ($P = 0.32$) in the presence of DFM. In terms of DFM acetate concentration was increased ($P < 0.01$) in the absence of MON and tended ($P = 0.07$) to decrease in the presence of MON. Monensin increased ($P < 0.01$) propionate concentration in the absence and presence of DFM. Butyrate concentration was decreased ($P < 0.01$) with the inclusion of monensin in the absence and presence of DFM, while DFM tended ($P = 0.07$) to increase butyrate concentration in the absence but not the presence of MON. Isobutyrate concentration was increased ($P < 0.05$) by MON in the absence of DFM but was unaffected ($P = 0.30$) in the presence of DFM. Direct-fed microbial alone and in combination with MON had

no effect ($P > 0.10$) on isobutyrate concentration. Similar to isobutyrate concentration, monensin increased ($P < 0.01$) isovalerate concentration in the absence, but not in the presence of DFM ($P = 0.72$). Additionally, DFM had no effect ($P = 0.42$) on isovalerate concentration in the absence of MON, but decreased ($P < 0.05$) concentration in the presence of MON.

Interactions between DFM and MON were absent for molar proportions of most VFA except for molar proportion of isovalerate ($P < 0.01$;Table 4.3). Additionally, a similar DFM x MON interaction tended ($P = 0.06$) to occur for molar proportions of isobutyrate. Molar proportions of isovalerate and isobutyrate were decrease ($P < 0.01$) with the addition of MON with and without DFM. Direct-fed microbial treatment decreased ($P < 0.01$) molar proportions of isovalerate and isobutyrate in the absence but not the presence of MON. Direct-fed microbial treatment effects were absent ($P > 0.10$) for molar proportion of acetate, propionate, butyrate and valerate. Monensin decreased ($P < 0.01$) the molar proportions of acetate and butyrate but increased ($P < 0.01$) the molar proportion of propionate. Valerate was unaffected ($P = 0.98$) by MON treatment. Acetate:propionate ratio was decreased ($P < 0.01$) with the inclusion of MON and was unaffected by DFM treatment (Table 4.3).

Ammonia-N concentration was increased ($P < 0.01$) by DFM and was unaffected ($P = 0.75$) by MON (Table 4.3). Both DFM and MON treatment increased ($P < 0.01$) ruminal pH levels (Table 4.3).

DISCUSSION

In vitro gas production techniques simulate the rumen environment allowing for characterization of treatment-mediated changes within the rumen and characterization of the kinetics of fermentation (Getachew et al., 1998). A positive relationship between extent of feedstuff degradation and *in vitro* gas accumulation is indicated by the increase in total gas production accompanied by an increase in substrate disappearance (Theodorou et al., 1994). Inclusion of DFM in the media increased both total and rate of *in vitro* gas production in the current experiment. However, these increases were either partially or totally abated by MON. To date these novel observations are indisputable for lack of comparisons of combined use of DFM and MON within the literature.

Ionophores selectively inhibit the metabolism of gram-positive bacteria, which lack a protective outer membrane, and protozoa in the rumen. Monensin's ability to inhibit lactate-producing rumen bacteria, including *S. bovis* and *Lactobacillus* species, was observed by Dennis et al. (1981) in sensitivity and growth rate trials. Similar results were reported in which monensin significantly decreased the lactobacilli and enterococci counts in *in vitro* incubations of the crop contents of chickens not previously exposed to monensin treatments (Rada and Marounek, 1996). This research suggests that monensin may have an adverse effect on the DFM treatments in the present study given the DFM primarily consist of *Lactobacillus acidophilus* and *Enterococcus faecium.* Additionally, Van Nevel et al. (1977) reported that efficiency for both total and net microbial growth were significantly depressed by addition of monensin in an *in vitro* study as determined by measuring the incorporation of ^{32}P-labeled phosphate in microbial material, which is in agreement with other data obtained *in vitro* as well as *in vivo* (Van Nevel and

Demeyer, 1977a). These findings suggest that monensin has the potential to inhibit the effects of the DFM on gas production parameters given the gram-positive nature of the DFM and/or that monensin has the potential to affect the other bacterial strains that may be positively influenced by DFM.

Direct-fed microbial stimulation of total gas production is indicative of an increase in substrate degradation and increased ruminal fermentation in the present study and is in contrast to those results reported by Kenney (2013) and Baah et al. (2009). Application of DFM (the same DFM as used in the present study) *in vitro* with a high concentrate total mixed ration substrate resulted in decreased total gas production (Kenney, 2013). Additionally, Baah et al. (2009) observed no differences in total gas production at 6, 24 and 48h and a linear decrease after 12h of incubation, with inclusion of increasing levels of *Lactobacillus casei* and *Lactobacillus lactis* (DFM), in 10:90 (forage:concentrate) barley grain based finishing diet. Furthermore, Baah et al. (2009) also observed no differences in total gas production at 6, 24 and 48h and a linear increase after 12h of *in vitro* gas production with inclusion of the previously mentioned DFM in 80:20 (forage:concentrate) barley silage based backgrounding diet. Additionally, no differences in total gas production, after 30h of *in vitro* fermentation, were observed with inclusion of DFM in high forage substrate (Experiment 1). Differences in observations within the literature when compared to the current study suggest that DFM (particularly lactate producing bacteria) effects on gas production are partly dependent on amount of time of incubation and substrate fermented. It is also possible that effects are dependent on the particular species or strains of bacteria, as well as does rates.

While monensin abated DFM effects on total gas production in the current experiment, when included alone, MON had no effect on total gas production of a high concentrate substrate. Despite the current findings, monensin-mediated changes in total gas production have been observed by others. Quinn et al. (2009) and Callaway and Martin (1996) observed a decrease in total gas production when compared to control, with inclusion of monensin *in vitro* with high concentrate substrates. Additionally, similar results were observed with inclusion of monensin in high forage substrates wherein MON decreased both total gas production and rate of gas produced (Experiment 1).

Gas production has been shown to be an indication of quantitative VFA production (Getachew et al., 2004). There was a positive relationship in the present study between gas production and total VFA concentration in response to DFM treatment in the absence of MON, although this was not the case in the presence of MON. This is suggestive of increased ruminal fermentation by means of DFM, but this increase may be abated by the presence of MON. Adverse effects of MON on DFM treatment have been previously discussed and are additionally supported by these findings.

Increase of total VFA concentrations in the presence of DFM alone are in contrast to other observations in literature. *In vitro* inclusion of DFM treatment with a mixed bacterial culture of lactate producing bacteria, the same DFM used in the present study, resulted in a tendency for decreased total VFA concentration (Kenney, 2013). Conversely, Baah et al. (2009) observed a linear increase in total VFA production, as compared to control, with provision of increasing levels of *Lactobacillus casei* and *Lactobacillus lactis* after 6 and 12 h of in vitro fermentations; however, no change was

observed at 24 and 48h fermentations. Additionally, DFM treatment, consisting of *Propionibacterium* and *Enterococcus,* had no effect on total VFA concentrations *in vivo* with inclusion of in steam-rolled corn based concentrate diets (Ghorbani et al., 2002; Beauchemin et al., 2003). Responses of ruminants to dietary supplementation of DFM vary among reports and have been attributed to differences in DFM sources, diet composition and animal physiological stages (Baah et al., 2009).

Modification of acid production as well as change in gas production are both widely accepted system modes of action of monensin (Schelling, 1984). Previous research by Richardson et al. (1976) and Ponce et al. (2012) observed decreased total VFA concentrations *in vitro* with inclusion of monensin treatments in high concentrate corn based diets. These *in vitro* results are supported by findings *in vivo* where total VFA concentrations were decreased with the inclusion of MON in similar high concentrate corn based diets (Raun et al., 1976; Richardson et al., 1976). Conversely, in a meta analysis of the effect of monensin dose on rumen volatile fatty acid profiles in high grain fed beef cattle, Ellis et al. (2012) reported no change in total VFA with monensin feeding. However, results from this analysis indicate that while there may not be significant changes in overall mean total VFA values, monensin has exhibited both positive and negative effects on total VFA concentrations within specific studies.

Although DFM altered total VFA concentration in the present study, it did not cause a shift in the molar proportions of major VFAs. Ghorbani et al. (2002) observed similar results to the present study where DFM (*Propionibacterium* and *Enterococcus)* had no effect on molar proportions of propionate and butyrate when included in a high concentrate diet of steers, however, in contrast DFM treatment increased molar

proportions of acetate. Additionally, molar proportions of acetate were increased with provision of *Lactobacillus acidophilus* alone or in combination with *Enterococcus faecium in vitro* with high concentrate substrate (Baah et al., 2009; Kenney, 2013). Furthermore, supplementation of *Enterococcus faecium* (EF) alone, on an 87% steam rolled barley based diet, increased the proportion of propionate and decreased the proportion of butyrate in rumen fluid compared to control (Beauchemin et al., 2003). Direct-fed microbial mediated changes in molar proportions of VFA have been observed in previous research although it is unclear why molar proportions were unaffected in the present study. Disparities could be due to subtle variability in diet composition between experiments within the literature.

It has been well documented throughout the literature that monensin has the ability to alter ruminal volatile fatty acid production and as such, modification of acid production is widely accepted as a system mode of action of monensin (Schelling, 1984). A highly accepted effect of monensin in the rumen is the decreased acetate to propionate ratio (Richardson et al., 1976; Quinn et al., 2009) as well as decreased molar proportions of butyrate (Ellis et al., 2012). Monensin treatment, in the present study, decreased molar proportions of acetate, butyrate, and isobutyrate and increased molar proportions of propionate. Similar to these findings, molar proportions of acetate were decreased by inclusion of MON *in vitro* with high concentrate based substrates, when compared with control and molar proportions of propionate were increased (Duff et al., 1995; Quinn et al., 2009; Ponce et al., 2012). The observed changes in acetate and propionate led to a decreased acetate:propionate ratio with inclusion of monensin when compared to control.

During ruminal fermentation methane is produced via methanogenic bacteria which utilize H_2, CO_2, and/or acetate (Bryant, 1979). Stoichiometry of the main anaerobic fermentation pathways indicates that ruminal production of acetate and butyrate via pyruvate produce H_2, while production of propionate via pyruvate utilizes H_2 (Bryant, 1979). Therefore, production of propionate can be considered a competitive pathway for hydrogen use, while acetate and butyrate promote methane production (Bryant, 1979). It has been observed that monensin decreased molar proportions of acetate as well as methane production *in vitro* with inclusion in a high-forage diet (Experiment 1). A direct measure of methane was not obtained in this study. However, the inclusion of monensin decreased the acetate:propionate ratio suggesting a decrease in methane production.

An inverse relationship between rumen pH and volatile fatty acid concentrations has been demonstrated across a variety of diets (Phillipson, 1942; Briggs et al., 1957). Moreover, ruminal acidosis is the result of the production of VFAs and lactic acid at a greater rate than ruminal clearance resulting in severe depressions in pH following feeding (Nagaraja and Titgemeyer, 2007). Because DFM supplementation has been shown to alter rumen pH and VFA profiles, it has been suggested as a preventative tool against ruminal acidosis. However, reports on the impact of DFM on ruminal pH are variable. Inclusion of the current DFM, *in vitro*, with a high concentrate substrate had no effect on pH (Kenney, 2013). Similarly, mixed cultures of lactate utilizing bacteria had no effect on mean, minimum, or maximum pH (Ghorbani et al., 2002; Raeth-Knight et al., 2007). Additionally, provision of *Enterococcus faecium*, *in vivo*, to cattle has been shown to have no impact on mean or maximum pH, but has reduced minimum daily pH

(Beauchemin et al., 2003). Dissimilar to previously presented observations, Nocek et al. (2002) observed an increase in mean daily pH in Holstein cows, in early lactation, receiving a combination of *Enterococcus faecium, Lactobacillus plantarum* and *Saccharomyces cerevisiae.* It is unlikely that the current observed increase in pH attributable to DFM is a function of total VFA concentration or molar proportions of VFA end products. Inclusion of DFM increased both pH of the culture broth and total VFA concentrations relative to control. These findings are similar to those observed using a forage based diet as substrate (Experiment 1). Additionally, DFM caused a similar increase in pH in the presence of MON despite wholesale changes of VFA molar proportions. It is possible that DFM decreased synthesis or increased utilization of lactate thereby increasing pH. Lactate was not measured in the present experiment, however this explanation is unlikely given the similar increase in pH that was observed with a high forages substrate (Experiment 1). Additionally, it is possible that the observed increased in NH_3 with DFM provision could be contributing the increase in pH.

Previous reports have demonstrated the ability of monensin to maintain a higher ruminal pH than non-treated controls during bouts ruminal acidosis (Nagaraja et al., 1981; Nagaraja et al., 1982; Burrin and Britton, 1986). With the increase in pH, Nagaraja et al. (1981) also reported decreased lactate concentrations with inclusion of 1.3 mg/kg bw monensin in cattle fed an 80:20 high forage diet where acidosis had been induced ruminally with glucose infusions. Additionally, Dennis et al. (1981) observed inhibited growth of major lactate producing bacteria with inclusion of monensin. Similar to DFM effects, monensin treatment increased ruminal pH levels in combination with increased total VFA concentrations in the present experiment. Lactate concentrations were not

measured in the present study. However, an increase in pH levels could be due to a decrease in lactate production. While monensin has been shown to increase pH levels, it is difficult to identify a mechanism to account for the observed increase in pH levels in conjunction with increased total VFA concentrations given the limitations of this experiment.

Ruminally degraded protein is converted to ammonia by ruminal microorganisms (Nolan, 1975). When carbohydrate is available as an energy source, ammonia can serve as a N source for microbial growth (Allison, 1969), however an imbalance between the rate of ammonia production and microbial growth can occur when an excessive ammonia accumulation occurs (Nocek and Russell). Monensin has been shown to reduce ammonia N concentration through inhibition of the hyper-ammonia producing bacteria, a small group of ruminal bacteria that are responsible for the production of most of the ammonia (Chen and Russell, 1989). While the inclusion of monensin in the present study had no effect on ammonia concentration, previous research has provided evidence of decreased ammonia concentrations, across a variety of diets, *in vitro* (Van Nevel and Demeyer, 1977b; Russell and Martin, 1984) and *in vivo* (Dinius et al., 1976; Poos et al., 1979; Yang and Russell, 1993) with monensin use. These effects of monensin were demonstrated by Yang et al. (1993) using switchback experiments. When animals (fed two different levels of soybean meal) were dosed with monensin, ruminal ammonia concentrations decreased within 5 days. Subsequently, the withdrawal of monensin treatments resulted in an immediate increase in ruminal ammonia concentrations regardless of feeding levels.

Ammonia concentrations increased with DFM treatments in the present study, which is inconsistent with previous work that has found no differences in ammonia-N

concentrations with DFM (lactic acid producers) provision *in vitro* and *in vivo* (Beauchemin et al., 2003; Kenney, 2013). Similarly, Ghorbani et al. (2002) reported no difference in NH_3 concentrations with inclusion of combined DFM treatment consisting of *Propionibacterium* P15 and *Enterococcus faecium* EF212 in steers fed a 10:90 (forage:concentrate) steam-rolled barley based diet. However, when *Propionibacterium* P15 was fed alone an increase in NH_3 concentration was observed (Ghorbani et al., 2002). Similarly increased ammonia concentrations to those observed by Ghorbani et al. (2002) were observed in an evaluation of two direct-fed microbial products, Bactozyme (primarily consisting of lactate producing bacteria) and Ru-max (combination of lactate producing bacteria, fungi and yeast) supplemented to sheep fed a 50:50 concentrate mixture and Egyptian clover based diet (Sallam et al., 2014). Disparities in observations within literature could be attributed to varying types of DFM and/or differences in nitrogen availability in feeds (Dawson, 1993). The observed increase in ammonia with DFM in the present experiment could be attributed to increased substrate degradation. This is consistent with the observed increase in gas production with DFM alone. However, this explanation is inconsistent with observed effects of DFM in combination with MON. Inclusion of MON partially abated total gas production but had no influence on DFM induced increase in ammonia concentration.

Table 3.1 Dry matter and nutrient composition of high concentrate *in vitro* substrate and donor diet

Ingredient	%, DM basis
Alfalfa, cube	20.00
Cracked Corn	75.78
Soybean Meal	2.00
Fat	0.20
Urea	0.62
Limestone	1.10
Trace Mineral-Salt	0.30
Vitamin A,D & E Premix	0.002
Nutrient, DM basis[a]	20%Forage:80%Concentrate
Crude Protein, %	13.03
Acid Detergent Fiber, %	26.11
Neutral Detergent Fiber, %	34.65
NFC, %	13.60
Calcium, %	1.11
Phosphorus, %	0.17
Magnesium, %	0.15
Potassium, %	0.97
Sodium, %	0.08
Sulfur, %	0.15
Iron, ppm	125.96
Zinc, ppm	21.20
Copper, ppm	7.30
Manganese, ppm	28.02
Molybdenum, ppm	1.06
NE_m, Mcal/kg	1.03
NE_g, Mcal /kg	0.57

[a] Based on analyses by Dairy One, Ithaca, NY.

Table 3.2 Effect of direct-fed microbial and monensin on rate and extent of gas production

	Treatment					P-Value		
	(-) DFM		(+) DFM					
	(-) MON	(+) MON	(-) MON	(+) MON	SEM	MON	DFM	DFM*MON
Plateau, mL	174.77[a]	173.74[a]	190.42[b]	179.11[c]	1.36	<0.01	<0.01	<0.01
Rate, hr^{-1}	0.183[a]	0.186[a]	0.188[b]	0.186[a]	0.002	0.60	0.11	0.07

[a,b,c,] Within a row, means that do not have a common superscript differ (P<0.05).

Table 3.3 The effect of direct-fed microbial and monensin on *in vitro* endpoint metabolites of a high concentrate substrate

	Treatment					P-Value		
	(-) DFM		(+) DFM					
	(-) MON	(+) MON	(-) MON	(+) MON	SEM[1]	MON	DFM	DFM*MON
Total VFA, mM	92.02[a]	103.39[b]	97.04[c]	98.76[c]	1.61	<0.01	0.90	<0.01
Acetate	52.43[a]	57.21[b]	56.10[b]	54.79[ab]	0.93	0.07	0.51	<0.01
Propionate	21.15[a]	28.34[b]	22.33[a]	26.92[b]	0.53	<0.01	0.82	0.02
Isobutyrate	1.46[a]	1.54[b]	1.51[ab]	1.47[ab]	0.02	0.38	0.80	0.02
Butyrate	10.41[a]	8.98[b]	11.05[a]	8.65[b]	0.24	<0.01	0.52	0.05
Isovalerate	3.74[a]	4.04[b]	3.86[ab]	3.82[a]	0.08	0.09	0.54	0.03
Valerate	3.94[a]	4.40[b]	4.12[c]	4.18[c]	0.07	<0.01	0.78	<0.01
Molar Proportion, moles/100moles								
Acetate	56.26	54.64	56.42	54.76	0.13	<0.01	0.30	0.87
Propionate	22.56	27.13	22.60	27.03	0.07	<0.01	0.67	0.35
Isobutyrate	1.58	1.48	1.54	1.47	0.01	<0.01	0.04	0.06
Butyrate	11.28	8.61	11.20	8.62	0.03	<0.01	0.39	0.22
Isovalerate	4.04[a]	3.88[b]	3.93[c]	3.87[b]	0.02	<0.01	<0.01	<0.01
Valerate	4.25	4.23	4.20	4.22	0.02	0.98	0.12	0.47
NH3, mmol/L	21.39	21.34	23.08	23.47	0.51	0.75	<0.01	0.67
Acetate :Propionate	2.66	2.05	2.66	2.06	0.01	<0.01	0.23	0.75
pH	6.32	6.37	6.45	6.5	0.01	<0.01	<0.01	0.94

[1] n=4/treatment

[a,b,c,] Within a row, means that do not have a common superscript differ (P<0.05).

CHAPTER 4: CONCLUSION

Direct-fed microbials are defined by the FDA as a source of viable microorganisms and these can include single bacteria, a combination of multiple strains of bacteria as well as yeast. Monensin is a carboxylic polyether ionophore that selectively inhibits the metabolism of gram-positive bacteria. Both feed additives have been shown to increase efficiency and weight gain in cattle as well as alter rumen fermentation. While monensin is widely used in receiving/finishing cattle rations as well as some forage-based operations, use of direct-fed microbials is not as prevalent. The novelty of these experiments lies in the exploration of possible interactions between a DFM containing a mixed culture of lactate producing bacteria, consisting primarily of *Lactobacillus acidophilus* and *Enterococcus faecium*, and monensin on *in vitro* ruminal fermentation.

Decreased substrate degradation by inclusion of monensin in a high forage substrate, was alluded to by the decrease in gas production and total VFA concentration. While DFM had no effect on gas production, total VFA concentration was slightly increased with direct-fed microbial treatment in high forage substrate in the absence but the not presence of MON. Monensin has the potential to inhibit the effects of the DFM (given the gram-positive nature of the DFM) and/or to affect the other bacterial strains that may be positively influenced by DFM. The observed increase in both pH and total VFA with inclusion of DFM is difficult to explain, because of the generally observed inverse relationship between pH and total VFA concentration. Nevertheless, the DMF-induced increase in pH does not appear to be the result of shifts in ammonia

concentration or molar proportion of VFA. Monensin decreased methane production and increased molar proportions of propionate. This is consistent with the concept that production of propionate can be considered a competitive pathway for hydrogen use, while acetate and butyrate promote methane production.

In the experiment using a high concentrate substrate, both total and rate of gas production was increased with DFM alone, but this increase was abated by the presences of monensin. This suggests that supplying DMF alone increased substrate degradation. In contrast, addition of MON increased total VFA concentrations in the absences but not in the presence of DMF. Combined these findings suggest that the beneficial effects of both DFM and MON, in terms of ruminal energy supply to the animal, may be lost when fed in combination. Positive additive effects were observed in terms of ruminal pH with additions of both MON and DFM, thereby suggesting that they can be used as a means to decrease the effects of sub-acute ruminal acidosis. The observed increase in ruminal pH in addition to increases in total VFA concentrations do not support the inverse relationship between pH and volatile fatty acid production that is commonly observed in literature. It is not likely that changes in pH are attributed to changes in total VFA or acid production given the increase in pH with and without changes in molar proportions of acids.

The results of this study indicate that the effects of this particular mixed culture of lactate producing bacteria and MON on *in vitro* fermentation are dependent on the substrate being fermented. It is likely that the observed effects of DFM on the concentrate substrate, as compared to the forage substrate, are due to these particular strains of DFM being lactate utilizing bacteria and that they would differ with use of

different bacteria. The observed interactions between direct-fed microbials and monensin provide a basis for further research to determine if monensin directly affects DFM bacteria or whether it opposes the actions of DFM on the rumen microbota.

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