ABSTRACT

Title of Dissertation: EVALUATION OF A METHOD TO MEASURE VOLATILE FATTY ACIDS AND GASES IN VITRO WITH MATHEMATICAL MODELING

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Methane emissions from ruminants have become an issue over the last 50 years. Previous research has shown that methane emissions are stoichiometrically linked with volatile fatty acid (VFA) profiles in ruminant animals. For example, a shift from acetate to propionate may decrease carbon dioxide (CO_2) and hydrogen (H_2) production, and in

turn, decrease conversion of CO_2 and H_2 to methane. In vitro methods have been developed to measure the digestibility of feeds, but such methods may not accurately estimate methane or volatile fatty acid (VFA) profile. The development of in vitro methods to accurately estimate gas production and VFA profile in rumen fermentation would enable isolation of fermentation effects from various animal interactions. Therefore, the focus of this dissertation was to develop an in vitro method that will have the same VFA and gas profiles as in the rumen. The objectives of this project are: to develop an in vitro technique that mimics an in vivo rumen environment in order to study VFA profiles and gas production during fermentation, to examine and evaluate the efficacy of selected feed additives (e.g. probiotics) on VFA profiles and gas production, and to develop a mechanistic model of the in vitro fermentation system and the effects of feed supplements on the system. The results indicate that gas profile, VFA profile, and gas production were affected by differing in vitro fermentation conditions (buffering capacity, headspace gas composition, acetate concentration). A review of the literature was conducted to establish the effect of probiotics such as lactic acid bacteria on in vitro and in vivo systems. These findings indicated *Enterococcus* and *Lactobacillus* species tended to affect ruminal fermentation parameters. Further in vitro analysis of these probiotics indicated these bacteria tended to affect ruminal fermentation, such as gas and VFA production. A developmental mechanistic model was built to predict whether the effect of probiotics was thermodynamically or kinetically limiting. Future studies will further development of this simple model by using published literature for a meta-analysis that may aid in further interpretation of rumen fermentation regarding thermodynamic limits and maximal efficiency of key rumen fermentation reactions.

EVALUATION OF A METHOD TO MEASURE VOLATILE FATTY ACIDS AND GASES IN VITRO WITH MATHEMATICAL MODELING

by

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DEDICATION

For Ann,

thank you for always being there, thank you for showing

me how to be strong no matter the obstacle

I am honored to be your rock

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER ONE

LITERATURE REVIEW

INTRODUCTION

The rumen environment is vast and home to millions of different microbes. Rumen fermentation results in the production of three main volatile fatty acids (VFAs): acetate, propionate, and butyrate, primarily via the uptake of glucose derived from plant biomass. Enteric methane production from ruminants is becoming a major dilemma for greenhouse gas emissions. Methane emissions are stoichiometrically linked with VFA profiles. For example, a shift from acetate to propionate may decrease CO_2 and H_2 production, and in turn decrease conversion of CO_2 and H_2 to methane. The pathway that produces 2 acetate, 2 CO_2 and 4 H_2 molecules per molecule of glucose is the most thermodynamically favorable in the rumen, and therefore most glucose is utilized this way. Unfortunately, this pathway leads to downstream production of methane, which is a potent greenhouse gas and a waste of dietary energy. Since VFA and gas profiles are linked stoichiometrically, in vitro methods designed to mimic the rumen should result in similar CO_2 and H_2 production, and similar VFA profile, compared to in vivo conditions.

The ability to introduce microbes into the rumen that would drive synthesis toward butyrate or propionate could aid in the reduction of methane production or a decrease in methane emissions. Once we have developed and validated a model procedure to study digestion in the rumen, we will be able to investigate factors that affect the process and potentially decrease unwanted methane emissions. There is not much known about the mechanisms that regulate VFA synthesis and selection. The focus of this study is to develop a more cost effective way for measuring changes in VFA profile and methane production using in vitro techniques, to introduce the in vitro system to microbial challenges that may alter the VFA profile, and finally to use a modeling system in which to analyze these

fermentation balances and begin to predict what mechanisms can direct downstream synthesis away from methane formation while utilizing both the thermodynamics and kinetics of the rumen system. The ability to predict these systems can lead to feed supplementation methods that will aid in the reduction of methanogens.

Rumen fermentation, rumen environment, and rumen microbes

There are two distinct systems within the ruminant: the external environment surrounding the animal and the microbial environment of the rumen (Russell and Hespell, 1981). Ruminants are the most effective users of nutrient resources found in the world as they are able to digest fiber and produce microbial proteins from sources such as non-protein nitrogen (Chalupa, 1977). The rumen is reported to serve as home to about 10^{10} to 10^{11} viable bacterial cells and nearly 10^{6} viable protozoal cells per milliliter, and is an ideal environment for fermentation encompassing about a seventh of an ruminant's mass (Russell and Hespell, 1981), though observations in our laboratory have shown up to 10^{15} viable and culturable cells per milliliter. The degradation of starch, fibers, and proteins occurs as a result of ruminal fermentation (Kohn and Boston, 2000). During digestion, ruminal fermentation occurs and breaks food down in to short chain fatty acids that provide energy to the animal (Russell and Hespell, 1981). Dietary manipulations can cause change to the use of energy provided by feed given the same amounts of digestible energy (Sutton, 1985) as well as to volatile fatty acid (VFA) concentrations in the rumen (Sutton et al., 2003). There is a direct correlation of acetate to propionate ratio from fermentation in the rumen with the dietary forage: concentrate (F:C) ratio (Moss et al., 2000). Adding grain to the diet causes changes in fermentation due to the increased presence of starch (Christophersen et al., 2008).

Fermentation in the rumen can also be controlled by regulation of rumen pH and chemical interventions (Chalupa, 1977) During fermentation, hydrogen is produced during the breakdown of glucose to pyruvate, and further utilized in the synthesis of propionate and butyrate (Chalupa, 1977). Shifting fermentation from acetate to increased propionate and butyrate may lead to an increase in energy of fermentation end products (Chalupa, 1977). Research has shown that using chemicals that inhibit methanogenesis can lead to increased production of propionate and butyrate due to the redirection of hydrogen (Chalupa, 1977). Carbohydrates are found in plants and are broken down to smaller sugar molecules and further broken down via fermentation to acetate, propionate, butyrate, methane, and carbon dioxide (Russell and Hespell, 1981). Protein degradation also occurs in the rumen breaking down into ammonia, carbon dioxide, and other fatty acids (Russell and Hespell, 1981). The rumen environment has bicarbonate, phosphate, and various proteins that provide buffering in the rumen, however this buffer may not be as effective when there is an excess of acid produced during fermentation which then results in a drop in pH (Russell and Hespell, 1981).

In vitro techniques used to evaluate rumen fluid

Feed evaluation methods were designed to be a more cost effective approach in determining nutritive quality (Dijkstra et al., 2005). Previous studies (Goering and Van Soest, 1970) designed in vitro rumen procedures to focus on the digestibility of a sample. Primary methods have been designed for digestibility and total gas volume. Gas production is affected by fermentation and the presence of a bicarbonate buffer (Getachew et al., 1998). Gas measurement methods focus on either measuring gas at atmospheric pressure, or calculated gas in a fixed container (Getachew et al., 1998). There are four established

methods of measuring gas: the Hohenheim gas method (Menke's), Lipid displacement, Pressure transducer systems, and the Manometric method (Getachew et al., 1998). Although there are more methods for measuring total gas volume and digestion, there are currently no in vitro methods that measure the profiles of volatile fatty acids, gases, and methane effectively. One cannot assume that methods to measure digestibility would adequately reflect profile of VFA that would be obtained from feeding a certain feed. There is a need for an improved in vitro method that does not have digestibility as its primary focus, but instead has fermentation byproducts and fermentation profile shifts as its driving force. There is a need for a method that allows ruminology of methane to be studied. In addition to this, the system must allow us to study the factors that may affect the VFA profile and the gas profile (CH_4, H_2, and CO_2).

Greenhouse Gas Emissions

Increasing greenhouse gas concentrations are a growing concern. Studies have predicted that as a result of greenhouse gas emissions that in the next 15 years the world may become 1-2 °C warmer (Moss et al., 2000). With these possible increases animal and human health are of great concern due to the effects on the environment. Although carbon dioxide is considered to be a strong factor contributing to global warming, methane is also of primary concern (Moss et al., 2000). The presence of methane in the atmosphere was first discovered in the 1940's (Migeotte, 1948). Of all the greenhouse gas emissions, methane is the second leading source in the United States (Kebreab et al., 2008).

Methane concentrations have been increasing steadily over the years are a result of anthropogenic causes and account for nearly 70 percent of methane production (Moss et al., 2000). Approximately 20 to 30 percent of methane comes from fossilized deposits,

whereas 70 to 80 percent of the methane comes from atmospheric carbon sources such as wetlands, biomass, and enteric fermentation (Johnson and Johnson, 1995). More importantly, nearly two-thirds (~ 60 percent) of the anthropogenic sources of methane are derived from agriculture (Moss et al., 2000), with about 44 percent coming from livestock (Gerber et al., 2013) . Agriculture also accounts for 7.7 percent of the total U.S. greenhouse gas emissions (U.S. Environmental Protection Agency, 2014). In order to combat increasing methane production there is a need for reducing methane from enteric fermentation and livestock manure.

Methane production increases with greater fermentable energy, but there is a diminishing return because as rumen pH declines with higher fermentable energy, the methane production becomes a smaller portion of fermentable energy. The ratio of acetate to propionate is a sensitive indicator of the more variable acids that change as the VFA profile changes, with ratios ranging from 0.9 to 4 (Johnson and Johnson, 1995). With carbohydrates, the higher the amount of carbohydrates fed increases methane loss whereas increasing the digestibility of a diet decreases the amount of methane loss (Johnson and Johnson, 1995). Diets that increase the grain content in a whole crop wheat silage have been shown to decrease methane emissions (McGeough et al., 2010) when compared to grain silage. Methane production is affected by dietary carbohydrates (digestibility) and hydrogen supply which regulated methane production through volatile fatty acids (Johnson and Johnson, 1995). Carbohydrates also play a role in the microbes available in the rumen as well as the rumen pH (Johnson and Johnson, 1995). Ruminal microbes, protozoa in particular tend to have an effect on the ruminal environment in the presence of a high concentrate diet (Johnson and Johnson, 1995).

According to the United States Environmental Protection Agency (U.S. EPA) enteric fermentation emissions have increased by 0.2 percent during the time period from 1990-2013 (U.S. Environmental Protection Agency, 2014). Enteric fermentation accounts for 26 percent of methane emissions in the US (U.S. Environmental Protection Agency, 2014). In enteric fermentation methane (CH_4) is eructated or exhaled by the animal, primarily ruminants (U.S. Environmental Protection Agency, 2014). In ruminants, beef cattle and dairy cattle are responsible for 71 percent and 25 percent of methane emissions respectively (U.S. Environmental Protection Agency, 2014). Methane eructation results in a loss of 3 to 10 percent of a cattle's ingested energy (Johnson and Johnson, 1995). Methane eructation begins early in a cattle's life within the first 4 weeks after birth (Anderson et al., 1987), which is generally the time cattle are weaned from their dam. Enteric emissions by cattle is a cause for concern and there is a need for some treatment that may lead to a reduction in methane emissions. Currently there is no effective method that consistently reduces methane emissions due to eructation.

Probiotic, prebiotic, and synbiotic treatments in the gut

Probiotics

Probiotics are live microbials that are utilized in feed to benefit the health of an organism (Fuller, 1989). Key rules to focus on when using probiotics include: the probiotic must be beneficial to the host, a non-pathogenic, must be viable, come from the same species as the host, must be viable when being stored, and good sensory properties (Collins and Gibson, 1999). *Lactobacillus* and *Bifidobacterium* are the major genera that probiotics are made from (Sorokulova, 2013). Studies have shown that the genus *Bacillus* (one of the most predominant bacteria found in nature) are now showing potential to be utilized as

probiotics (Sorokulova, 2013). *Bacillus* have very high metabolic activity which is why they may be ideal for microbial gut function (Sorokulova, 2013). Subtilisin (a non-specific protease) and catalase (a common enzyme) have been shown to increase the viability and growth of lactobacilli, and are made by the *Bacillus subtilis* species (Hosoi et al., 2000). A limitation of synthesizing probiotics from the *Bacillus* genus is that the activity of the probiotic is species specific, meaning each species must be tested in order to determine if the strain can be used as a probiotic (Sorokulova, 2013). Two of the most common microbes used in feed are *Saccharomyces cerevisiae* an *Aspergillus oryzae* (Moss et al., 2000). Direct fed microbials (DFM) have become a primary focus in livestock as antibiotic resistance increases (McAllister et al., 2011). Direct fed microbials may have the ability to alter fermentation activity and shift fermentation to other byproducts in the rumen (McAllister et al., 2011). There have been several studies that have addressed the efficacy of different bacterial species in ruminal fermentation (McAllister et al., 2011).

Table 1.1 Direct fed microbial species from ruminant studies. Adapted from McAllister et al., 2011.

Table 1. Summary of published work and proposed modes of action for direct fed microbials (DFM) used in ruminant studies since 1991. The cited reference represents the latest work published on the DFM of interest in each livestock class

Bacteria	Application[zy]	Example references	Mode of action
Lactic acid producers			
Enterococcus faecium	C (1) D (8) F (4) L (1)	Nocek et al. (2003) Fleige et al. (2007) Emmanuel et al. (2007) Abas et al. (2007)	• stimulation of lactic acid utilizers • Competitive exclusion • Direct antibacterial effect • Enhanced immune response
Lactobacillus plantarum	C (3) D (4) F (4) L (1)	Aydin et al. (2009) Jatkauskas and Vrotniakien (2007) Nocek et al. (2002) Lema et al. (2001)	
Lactobacillus casei	D (3) L (1)	Yasuda et al. (2007) Lema et al. (2001)	
Lactobacillus acidophilus	C (1) D (1) F (3) L (1)	Al-Saiady (2010) West et al. (2005) Tabe et al. (2008) Lema et al. (2001)	
Rumen bacteria			
Megasphaera elsdenii	D (1) F (3) L (1)	Henning et al. (2010) Leeuw et al. (2009) Aikman et al. (2009)	• Increased propionate • Moderation of pH
Prevotella bryantii	D (1)	Chiquette et al. (2008)	
Selenomas ruminantium	L (1)	Wiryawan and Brooker (1995)	
Other			
Propionibacterium freudenreichii	F (4)	Vasconcelos et al. (2008)	• Increased propionate • Moderation of pH
Propionibacterium jensenii	C (2) D (1)	Adams et al. (2008) Francisco et al. (2002)	
Propionibacterium acidipropionici	C (1)	Kim et al. (2000)	
Bifidobacterium spp.	C (1)	Krehbiel et al. (2003)	• Lower tract function
Bacillus spp.	C (4) D (1) F (2)	Aydin et al. (2009) Qiao et al. (2009) Arthur et al. (2010)	• Substrate utilization
Escherichia coli	C (2)	Schamberger et al. (2004)	• Competitive exclusion
Yeast and Fungi			
Saccharomyces cerevisiae	C (4) D (14) F (9) L (2)	Kalmus et al. (2009) Liou et al. (2009) Thrune et al. (2009) Chaucheyras-Durand et al. (2010)	• Rapid establishment of microbial consortia in newborn • Improved fiber digestion • Enhanced lactic acid utilization • Oxygen scavenging • Unidentified growth factors/nutrients • Source of hydrolytic enzymes

One study (Henning et al., 2010) observed no difference in total VFAs produced but were able to observe an increase in propionate as well as a shift from propionate production to butyrate production, though this was dependent on external factors such as pH and substrate. Though this is an interesting finding they did not show if there was a decrease in acetate in response to the propionate increase. Propionate producing bacteria may serve as a competitor whether direct or indirect with bacteria that primarily lead to acetate

production. The use of lactic acid producing bacteria have a very important role in the potential to shift rumen fermentation. Lactic acid producing bacteria are believed to stimulate the growth of bacteria that use lactic acid, this group of bacteria are also called propionate producing bacteria (McAllister et al., 2011). *Propionibacteria* are naturally producers of propionate (Vyas et al., 2014). Conversion of lactate and glucose to the byproducts of acetate and propionate are two characteristics of *Propionibacterium* (Ghorbani et al., 2002). The use of *Propionibacterium* has been shown to decrease the risk of acidosis without affecting the pH of the rumen or the blood (Ghorbani et al., 2002).

Prebiotics

Prebiotics are a bit different from probiotics as they are not so much for the host but are feed supplements for the existing microbial species in the host. Prebiotics are non-digestible and function by increasing growth and activity of the host microbes in the colon (Gibson and Roberfroid, 1995). There are fewer key components to the use of prebiotics compared to probiotics. Prebiotics must be selective (specific microbial substrate) for the bacteria in the colon, it must not be absorbed in the upper GI tract, and must shift the microbial environment to healthier bacteria (Collins and Gibson, 1999). Lactate precursors are considered a primary resource to be utilized as prebiotics (Collins and Gibson, 1999).

Fructans, fructooligosaccharides (FOS), galactooligosaccharides (GOS), and xylooligosaccharides (XOS) are some of the most common prebiotics used (Scott et al., 2013). The structure of a fructan is a fructose polymer connected to a glucose terminal end (López and Urıas-Silvas, 2007). Fructan fermentation produces short chain fatty acids in the colon and are not susceptible to GI tract enzymes (Cummings et al., 2001). Inulins are carbohydrates so they occur naturally and have been shown to aid in digestive health

(Jackson and Taylor, 1999). FOS contain glucose and fructose and fermentation of them produces lactate, acetate, propionate, and butyrate (Bornet et al., 2002). Gas is also produced by FOS and they have the ability to reduce the growth of harmful bacteria (Bornet et al., 2002). Galactooligosaccharides are also carbohydrates that can increase *Lactobacillus* and *Bifidobacteria* production (Rowland and Tanaka, 1993). Xylooligosaccharides are made of xylose and have shown an affinity for *Bifidobacteria* leading to an increase in butyrate production (Lecerf et al., 2012). Long-chain inulin (another class of prebiotics) have been shown to increase butyrate production in fecal microflora (Kleessen et al., 2001). Another study (Dewulf et al., 2013) indicated in humans that mixing three classes of probiotics (long-chain inulin, short-chain FOS, and GOS) led to an increase in *Faecalibacterium prausnitzii*, a butyrate producing bacterium. Dewulf et al., 2013, were able to show that the response of the *Faecalibaceterium prausnitzii* to the prebiotic mix may indicate that other butyrate producing species, like *Firmicutes* may also benefit from this practice. Prebiotics have the ability to do two things: they can help increase the viability and abundance of probiotics and they can also lead to a decrease in harmful bacterial species like *Clostridium* (Riscuta, 2013). *Bifidobacterium* have been found to increase probiotic growth (Riscuta, 2013), while other studies (Koleva et al., 2012) have shown that FOS and inulin can reduce *Clostridium difficile* growth in rats.

Synbiotics

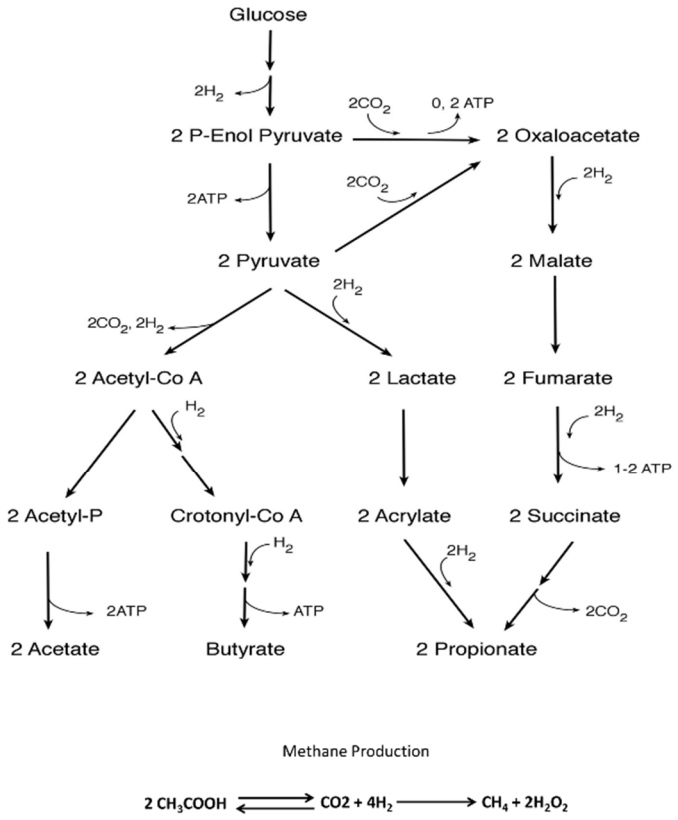
There is a third less often used class of direct fed microbials which combine prebiotics and probiotics into a single supplement called synbiotics (Collins and Gibson, 1999). The concept is derived from the potential to increase survival of probiotics by providing specific feed (prebiotics) for that organism (Collins and Gibson, 1999). Although

most studies have been conducted for human health or in mice and rats, it is possible that utilizing some of these supplements (especially those that promote butyrate or propionate) may improve fermentation in cattle and lead to decrease gas production.

Thermodynamics and biological modeling in rumen fermentation

All chemical reactions are a result of a combination of kinetic and thermodynamic reactions in a system (Chang, 1981). The second law of thermodynamics states that entropy increases in the universe will always increase over time and that in a reversible process will not be changed (Engel et al., 2012). The theory of enzyme kinetics states that the concentrations of substrates control of the rates of product formation, if all reactions are thermodynamically possible (Kohn, 2007). This limits the reaction rates to the available concentrations of substrate or enzyme in the system, thus being a kinetically controlled system. If these reactions are controlled by thermodynamics, then the reactions will be limited by the buildup of products relative to reactions, and these reactions will not occur (Kohn, 2007). The Michaelis-Menten equation is the primarily accepted and common equation used to quantify the rate of biological reactions (Chang, 1981). Kohn and Boston, 2000 state that degradation of ruminal products is metabolized to carbon dioxide (CO_2), methane (CH_4), microbial mass, volatile fatty acids (VFAs), and ammonia (NH_3). Thermodynamics is responsible for the pathway branches that are available for forming a product (Kohn and Boston, 2000). Previous studies have calculated the pathways of the breakdown and use of glucose during ruminal fermentation (Ungerfeld and Kohn, 2006).

Figure 1.1 Pathways of fermentation in the rumen. Methane production pathway.



Adapted from Ungerfield and Kohn, 2006

Some of the primary chemical reactions that affect the thermodynamics in a rumen system were identified by Ungerfeld and Kohn, 2006. Fluxes for the following chemical reactions were identified:

Figure 1.2. Chemical reactions and conversion of glucose to VFAs. Adapted from (Ungerfeld and Kohn, 2006).

$C_6H_{12}O_6$ (glucose) + $2H_2O$ ←→ $2CH_3COOH$ (acetate) + $2CO_2$ + $4H_2$

$C_6H_{12}O_6$ (glucose) + $2H_2$ ←→ $2CH_3CH_2COOH$ (propionate) + $2H_2O$

$C_6H_{12}O_6$ (glucose) ←→ $CH_3CH_2CH_2COOH$ (butyrate) + $2CO_2$ + $2H_2$

CO_2 + $4H_2$ ←→ CH_4 + $2H_2O$

$2CO_2$ + 4 H_2 ←→ CH_3COOH (acetate) + $2H_2O$

CH_3COOH (acetate) + $4H_2$ + CO_2 ←→ $CH_3C\ H_2COOH$ (propionate) + $2H_2O$

The stoichiometry of the system determines VFA production as well as gas formation (Ungerfeld and Kohn, 2006). In kinetics, reactions occur as a result of the presence of substrates or enzymes (Ungerfeld and Kohn, 2006). Most fermentation systems are usually near thermodynamic equilibrium (Kohn and Kim, 2015). The focus in biology has often been on the kinetics of fermentation, though current studies have shown that thermodynamics in rumen fermentation cannot be ignored (Kohn, 2014). Previous studies have shown that the ΔG (determines whether a reaction can proceed) for VFAs and some gases is almost 0, showing the reaction pathways are very close to equilibrium (Kohn and Boston, 2000). Descriptions of the rumen system have been portrayed by mechanistic modeling in order to explain fermentation (France et al., 1982; Baldwin et al., 1987). The model by France et al., 1982 was the first model that utilized pulse doses. The first rumen model incorporating thermodynamics was by Kohn and Boston, 2000. Other studies have

investigated models to evaluate gas production and found support for the Michaelis-Menten equation being more useful than other model types (Dhanoa et al., 2000). Studies have shown (Kohn and Boston, 2000; Kohn, 2003; Ungerfeld and Kohn, 2006; Kohn, 2007; Janssen, 2010; Kohn and Kim, 2015) the increasing need to more thoroughly investigate the effect of thermodynamics in ruminal fermentation processes. Using V_{max} (enzyme reaction rate), and K_{eq} (equilibrium constant) to determine the thresholds of different reactions will allow a starting point to understand when and why changes occur to the VFA and gas profile, and possibly enable us to use that information to develop a model that contains and utilizes both kinetic and thermodynamic reactions. We will also be able to calculate ΔG at a given time which will inform us how close a reaction is to equilibrium. The use of mechanistic and mathematical modeling will aid in furthering understanding and potentially being able to predict fluxes of the system as well as to account for dynamics that may lead to fermentation shifts.

Objective

The objectives of this project are: to develop an in vitro technique that mimics an in vivo rumen environment in order to study VFA profiles and gas production during fermentation, to examine and evaluate the efficacy of selected feed additives (e.g. probiotics) on VFA profiles and gas production, and to develop a mechanistic model of the in vitro fermentation system and the effects of feed supplements on the system. There are several factors and mechanisms that affect both VFA production and the gas profile in vivo. Factors to be considered when designing a method that compares to the in vivo model are the types of feed, the feeding frequency effects, and diurnal variation. The mechanisms behind VFA and gas profiles are the kinetics and thermodynamics of a system. The kinetics

considers the different microbial activities that can occur based on enzyme and substrate availability, and the thermodynamics considers that dependent upon microbial activity the threshold (V_{max}) required for methanogenesis is not only affected by the presence of the enzymes and substrate of the microbes, but also the amount of product present in the system at a given time. We aim to test if an in vitro method can provide comparable VFA and gas profile of an in vivo, as well as to determine the limitations of the in vitro model regarding thermodynamic and kinetic reactions in the system.

CHAPTER TWO

TEST OF CONDITIONS THAT AFFECT IN VITRO PRODUCTION OF VOLATILE FATTY ACIDS AND GASES[1]

L. M. JUDD and R. A. KOHN

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ABSTRACT: In vitro methods have been developed to measure digestibility, but such methods may not accurately reflect gas production or volatile fatty acid (VFA) profile. The objective of this study was to determine the effect of different in vitro conditions on VFA and gas production. Experimental design was a 4 × 2 × 2 factorial CRD with 4 replicates. Treatments were 4 ratios of medium to rumen fluid by volume (5:95, 25:75, 50:50, 75:25), 2 concentrations (w/v) of added timothy hay (0.5% or 1%), with or without added sodium acetate (increased initial concentration by 50 mM). Total volume of medium and rumen fluid was 10 mL per tube. Measurements of gas production and VFA were recorded at 0, 4, 16, 24, and 48 hours. Statistical analyses used a mixed model including all fixed effects and interactions with tube as a random effect, and time nested within tube. Total gas production increased ($P < 0.001$) with higher medium proportion. The final pH increased ($P < 0.0001$) as medium proportion increased. Medium proportion positively affected ($P < 0.05$) overall average concentration of both acetate production and propionate production. Higher hay concentration increased ($P < 0.0001$) total gas produced from 0 to 48 hours, increased total acetate production ($P < 0.01$), propionate production ($P < 0.001$), and decreased pH between 24 and 48 hours ($P < 0.0001$). Sodium acetate addition increased ($P < 0.0001$) pH between 24 and 48 hours. Acetate:propionate (A:P) concentration decreased over time ($P < 0.0001$). Initial rumen fluid A:P ratio was 3.7 but average A:P ratio of produced VFA started at 2.2 and increased to 2.50 (SE = ± 0.51). The A:P ratio differed for VFA produced in vitro compared to initial rumen fluid, but no tested treatments were found to change A:P ratio.

Key words: fermentation gases, in vitro procedures, methane, rumen fermentation, sodium acetate, volatile fatty acids

INTRODUCTION

Increasing greenhouse gas emissions are a growing concern. Agriculture accounts for 7.7 percent of total U.S. greenhouse gas emissions (U.S. Environmental Protection Agency, 2014). Of all the greenhouse gas emissions, methane is the second leading source in the United States (Kebreab et al., 2008). Among ruminants in the US, dairy cattle and beef cattle are responsible for 25% and 71% of enteric methane emissions respectively (US EPA, 2014). Nearly two-thirds (~ 60 percent) of the anthropogenic sources of methane in the world are derived from agriculture (Moss et al., 2000), with about 44 percent of global anthropogenic methane emissions coming from livestock (Gerber et al., 2013).

Although the most reliable measurements of enteric methane and carbon dioxide emissions are from animals placed in chambers, development of in vitro methods would facilitate replication of multiple treatments and enable isolation of fermentation effects from animal interactions. In vitro methods have been developed to measure digestibility, but such methods may not accurately reflect gas and VFA production. Available in vitro methods focus on the digestibility of a sample (Goering and Van Soest, 1970), or cost-effective feed evaluation to determine nutritive quality (Dijkstra et al., 2005).

Rumen fermentation results in the production of three main volatile fatty acids (VFA): acetate, propionate, and butyrate, primarily via the conversion of glucose derived from plant biomass. Gas production is stoichiometrically linked with VFA profiles. For example, most glucose is fermented through a pathway that produces 2 acetates, 2 CO_2 and 4 H_2 molecules per molecule of glucose, and the H_2 is used to convert glucose into propionate, or CO_2 and H_2 are used to make CH_4. Whereas the fermentation pathways are

limited in part by thermodynamics (Ungerfeld and Kohn, 2006), the profile of products (e.g. gases or VFA) could influence subsequent pathways.

The goal of this research was to develop an in vitro technique to study ruminal metabolism related to methane and VFA production. This first study compares different conditions of the fermentation on VFA and gas production. We hypothesize that addition of different substrates or products (e.g. feed, acetate, and buffer) could bring about changes in the profile of products formed. For example, we hypothesize that addition of acetate into a rumen fermentation medium will shift fermentation away from acetate and toward propionate and butyrate in accordance with thermodynamic control.

MATERIALS AND METHODS

Experimental procedures were approved by the University of Maryland Institutional Animal Care and Use Committee (IACUC) [398173-1].

Experimental Design and Treatments

The experimental design was a 4 × 2 × 2 factorial CRD; the treatments were ratio of medium:rumen fluid (calculated by volume at ratios of 5:95, 25:75, 50:50, and 75:25), Timothy hay amount (0.05 vs. 0.10 g), and addition of sodium acetate or not (NaOAc). Each treatment was replicated 4 times. Total volume of medium and inoculum was 10 ml per 20-ml Hungate tube.

Rumen Fluid Collection and Sample Preparation.

Rumen fluid was collected from a permanently non-lactating rumen-cannulated cow consuming a timothy hay diet and was prepared according to Goering and Van Soest

(1970). Approximately 0.5-1 L of rumen contents (solids and liquid) were collected anaerobically in 50-mL centrifuge tubes. Rumen fluid was blended for 20 seconds under CO_2 and was strained through 4 layers of cheesecloth and glass wool into a 1-L flask infused with CO_2.

Timothy hay was pre-weighed (0.05 g or 0.10 g) into labeled 20-mL Hungate glass tubes with rubber stoppers and screw caps. The Timothy hay was measured on a Mettler Toledo AE260 Delta Range (Columbus, OH) 4-place balance. Average weight for the 0.5 g Timothy hay and 0.10 g Timothy hay were 0.053 g (SD = 0.001) and 0.104 g (SD = 0.002), respectively. In vitro buffered medium was prepared, perfused with CO_2, and reduced with reducing agent as previously published (Goering and Van Soest, 1970). The Hungate tubes were perfused with CO_2 while the different amounts of buffered medium were added according to each treatment in random order. Each tube receiving added acetate treatment received 0.5 ml of 1 M sodium acetate (increasing starting concentration of acetate in these treatments by 50 mM), and those tubes without added acetate received an additional 0.5 ml in vitro medium. Processed rumen fluid (as described above) was added to each tube in random order, while infusing tubes with CO_2, and stirring the rumen fluid using a magnetic stir bar. Each tube was then sealed with a stopper and screw cap and inverted. A 20-mL gas-tight syringe and needle were inserted into the rubber stopper at the top of each tube for measurement of gas production. The 20-mL syringes had tick marks at 0.2-mL intervals. Tubes were subsequently incubated at 39° C.

Gas was removed and liquid in each tube was sampled before placing the samples into the incubator (39°C). Liquid samples for volatile fatty acids (VFAs) were collected by inverting the in vitro tube, allowing the substrate to settle and using a 27-gauge needle and

syringe to withdraw 1 mL of sample. The sample was then expelled into a 1.5 mL microcentrifuge tube and frozen in a -20°C freezer for further analysis. There was no correction for removal of fluids as this could impose a bias on the concentrations as existing VFA and some substrate would be removed. Non-lactating and lactating cows have mean retention times of approximately 20 to 24 hours on high concentrate diets, and approximately 30 hours on high forage diets, (Hartnell and Satter, 1979; Ishler et al., 1996); thus measurements of gas production and VFAs were recorded at 0, 4, 16, 24, and 48 hours.

Gas Measurement

Gas production was measured at 39˚C in mL and converted to μmol by dividing the average gas produced for each treatment by 25.6 mL/mmol and multiplying by 1000 μmol/mmol per the ideal gas law. Gas was recorded at each timepoint, the produced gas was then expelled from the syringe, and the syringe was screwed back onto the needle. The non-CO_2 gas was measured at 48 hours by expelling produced gas from syringe into a Wheaton bottle containing 40 mL of 6N NaOH. The bottle was vigorously shaken for 30 seconds and the remaining gas was measured by allowing the syringe to expand. The values were recorded as a ratio of non-CO_2 to total produced gas per sample and converted to μmol units. Previous experiments found using gas chromatography that nearly all fermentation gas was either CO_2 or methane.

VFA Analysis

The VFA samples were prepared using a modified Erwin et al. (1961) method. VFA samples were thawed at room temperature then spun in a centrifuge at 12,000 × g for 30 minutes at 4°C. Phosphoric acid (10% H_3PO_4) was added to the supernatant of each sample.

VFA concentrations were measured using gas chromatography (Hewlett-Packard model 6890) with a 4.6 m length x .318 cm outer diameter. x 2.1 mm inner diameter packed GC column (60/80 Carboxen-1000 support, model 1-2390, Supelco, Inc, Bellefonte, PA), and flame ionization detector (FID). The split ratio of the injector port (220°C) was 100:1. Helium was used as a carrier gas with a flow of 40 mL/min. The initial column temperature was 130°C held for 10 min, then increased to 200 °C (ramp of 80°C/min) for 1 min, and a post-run temperature of 120°C. The detector temperature was 200°C with a hydrogen and air flow of 40 mL/min and 200 mL/min, respectively. VFA production is reported as the change in concentration at each interval.

Statistical Analysis

Statistical analyses were conducted using JMP Pro 11 (JMP®, Version *11*. SAS Institute Inc., Cary, NC, 1989-2007). Two mixed models were used. The first was a mixed model: $y_{ijklm} = \mu + H_i + B_j + A_k + T_l + \gamma_{ijkl(m)} + \varepsilon_{ijklm}$ for response variables measured over time within tubes. The second model was: $y_{ijkl} = \mu + H_i + B_j + A_k + \gamma_{ijk(l)} + \varepsilon_{ijkl}$ where, $y_{ijk(l))}$ for response variables measured only once. For each model Y is the response, μ is the mean of the population, H_i is the effect of hay, 0.05 or 0.10 grams, B_j is the effect of the buffer/rumen fluid concentration, at levels of 5:95, 25:75, 50:50, and 75:25, A_k is the effect of acetate, with or without 50 mM addition, T_l is time measured at 0, 4, 16, 24, and 48 hours, $\gamma_{ijk(l)}$ is the random effect of the tube nested in treatment (hay, buffer, and acetate combinations or hay, buffer, acetate, and time), and $\varepsilon_{ijkl(m)}$ is the residual effect. All interactions were included in each model and time was continuous. This model measured the effect of treatment on total gas production, pH, and VFA production over time.

RESULTS AND DISCUSSION

Effect of Buffered Media

Increasing ratio of medium:inoculum increased gas production after 4 h and resulted in higher total gas production (Table 2.1). Gas production is affected by fermentation and the presence of bicarbonate buffer (Getachew et al., 1998). Our results differed from a study in which the amount of gas produced increased as the concentration of rumen fluid increased (Rymer et al., 1999). We found the opposite effect. Rumen fluid in the cow is usually equilibrated with less than 1 atmosphere CO_2 because of the presence of methane, but the in vitro buffer we used was equilibrated with 1 atm CO_2. Thus, having more bicarbonate buffer would allow for more CO_2 to be released (Kohn and Dunlap, 1998).

The gas collected at the end of the fermentation comprised original CO_2 that was not flushed out and produced gases. We measured the fraction of the final gas that was CO_2 and attributed the remainder to the non-CO_2 fraction. In previous in vitro experiments, we have observed that nearly all the non-CO_2 gas in the fermentation is methane. Both the non-CO_2 gas and CO_2 gas numerically increased with higher medium percentage (Table 2.1). If the main reason for an increased gas production from the higher proportion of medium was merely the evolution of CO_2 from buffer, we would have expected the non-CO_2 fraction to have been diluted. The non-CO_2 fraction did not decrease in the treatments with more medium, and the increase in gas production occurred increasingly at later time points, suggesting that evolution of CO_2 from buffer may not be a complete explanation for why gas production was higher in treatments with more media.

The pH at 48 h increased as buffer concentration increased, resulting from greater buffering capacity of the treatment with higher ratio of medium:inoculum (Table 2.1). This increase in pH is in agreement with findings from other studies (Tripathi et al., 2004; González et al., 2008). Tripathi et al. (2004) found pH to range from 6.03 (0% bicarbonate) to 6.44 (2.25 % bicarbonate), whereas in González et al. (2008), pH ranged from 5.91 (0% bicarbonate) to 6.38 (5 % bicarbonate). The pH in this study is higher, compared to another study (Erdman, 1988), which may be due to the greater buffering capacity of treatments with higher ratio of medium:inoculum. It is possible in the present study that the treatments with higher ratio of medium:inoculum had higher pH as a result of the presence of the sodium bicarbonate and less rumen fluid. Studies have shown that when pH is lower than 6.0 the buffering capacity for bicarbonate is reduced due to having an effective pKa of 6.7 (Terry et al., 1969; Russell, 1998). The tubes with more rumen fluid had lower pH than tubes with less rumen fluid and this may have resulted from a slight reduction in buffering capacity for those.

VFA production was calculated as the change in concentration at each interval for each treatment. Acetate and propionate production (mM) increased ($P < 0.05$) as media concentration increased (Table 2.1). Initial acetate concentrations were: 59, 55, 47, and 38 mM (SE ± 0.8) for the 5, 25, 50, and 75% medium treatments, respectively. The initial propionate concentrations for the 5, 25, 50, and 75 % medium treatments were: 13, 12, 10, and 8 mM (SE ± 0.2). These results are similar to findings that showed increasing ratio of medium:inoculum also increased production of volatile fatty acid (González et al., 2008). Table 2.2 shows VFA production over time for the buffer treatment. There was an effect ($P < 0.01$) of buffer on acetate production between 4 and 16-h and between 24 and 48-h.

Propionate production increased (P < 0.01) from 4 to 16-h, and between 16 and 24 h. There was no effect on butyrate production, acetate:propionate (A:P) ratio, or acetate:butyrate (A:B) ratio over time by the buffer treatment.

Effect of Hay

As expected, gas production was greater ($P < 0.01$) with the higher concentration of hay in the tube (Table 2.3), but gas production (μmol/g) per unit hay was higher ($P < 0.05$) for the lower concentration of hay. Since gas is produced from both the hay substrate and additional substrate from the rumen fluid and dividing by hay only corrects for the amount of hay, a higher gas/hay was expected for the lower hay concentration.

Between 24 and 48-h pH decreased ($P < 0.01$) with increased hay concentration (Table 2.3) as expected since more acid would be produced from the greater amount of substrate.

Acetate production (mM; Table 2.3) and propionate production (mM; Table 2.3) increased ($P < 0.01$) as hay concentration increased. Initial acetate and propionate concentrations at 0.5 g and 0.10 g Timothy hay were 48 and 50 mM (acetate; SE ± 0.6) and 9.7 and 11.8 mM (propionate; SE ± 0.13). Table 2.4 illustrates the effect of hay on VFA production over time. Acetate production was higher ($P < 0.05$) with the higher hay concentration (Table 2.4) and propionate production (Table 2.4) also increased ($P < 0.05$) with increasing time interval and was highest with the higher concentration of hay. There was no effect of the concentration of hay on total VFA, A:P or A:B ratio over time. Studies have shown that the form of digestible energy can affect the volatile fatty acid (VFA) concentrations in the rumen (Sutton et al., 2003).

Effect of Acetate

The addition of acetate was hypothesized to shift fermentation away from acetate, which might have decreased acetate production and concomitant gas production. Acetate addition did not affect total gas production (Table 2.5), and surprisingly increased gas production between 24 to 48 h ($P < 0.05$).

The addition of 50 mM sodium acetate (Table 2.5) increased ($P < 0.01$) pH from 24 to 48-h, also as expected because the acetate salt (pKb = 9.25) acts as an additional buffer. At lower pH, the use of hydrogen for propionate production could decrease the availability of hydrogen for methane production (Johnson and Johnson, 1995; Janssen, 2010; Zijderveld et al., 2010). Added acetate can decrease acetate production by thermodynamics. Additionally, the acetate itself could be interconverted to other VFAs or methane (Ungerfeld and Kohn, 2006).

Although A:P concentrations decreased ($P < 0.01$) over time (data not shown), there was no effect of acetate addition on the production ratio of A:P, or A:B over time (Table 2.6). These findings were contrary to our hypothesis that addition of acetate would lead to a shift in fermentation away from acetate and towards propionate and butyrate and that fermentation conditions will affect the ratio of produced VFA and gas profiles. One might expect in the presence of sodium propionate or sodium butyrate that VFA profiles (overall concentrations) would differ from these findings. Sodium propionate may increase the A:P ratio by decreasing propionate production and sodium butyrate may increase the A:B ratio by decreasing butyrate production. The initial rumen fluid A:P ratio was 3.7 but the A:P ratio of VFA produced averaged 2.5 (SE = ± 0.51).

Effect of Treatment Interactions

There was a tendency ($P < 0.10$) for an interaction between acetate treatment by ratio of medium:inoculum treatment on gas production between the 24 to 48-h interval. Gas production between 24 to 48-h increased with acetate addition.

There was an interaction ($P < 0.01$) of hay with the ratio of medium:inoculum on total gas production (Fig. 2.2A) and a tendency ($P < 0.10$) on gas production between 24 to 48-h (Fig 2.2B). As the concentration of timothy hay doubled and medium proportion increased, total gas production and gas production between 24 and 48-h increased. Total gas increased ($P < 0.01$) as ratio of medium:inoculum increased in the higher hay concentration compared to the lower hay concentration.

There was an acetate treatment by hay concentration interaction on pH (Fig. 2.3A). The lower hay concentration had higher average pH with added acetate than without. There also was an interaction of ratio of medium:inoculum with acetate addition (Fig. 2.3B) on pH ($P < 0.01$). The pH was lower without acetate addition for the low ratio of medium:inoculum. The pH was also affected by the buffer by hay interaction (Fig. 2.3C) with ratio of medium:inoculum, and was depressed more for the high concentration of hay when the ratio of medium:inoculum was low. There would be a greater effect of bicarbonate buffering in the treatment with higher ratio of medium:inoculum when there was a higher concentration of hay and more need for buffering.

Fermentation may be regulated by kinetic control when concentrations of products are limited, and activities of substrates and enzymes determine rates of individual reactions and profile of products (Ungerfeld and Kohn, 2006; Kohn, 2007). Most fermentation

systems, however, are often near thermodynamic equilibrium in which the product accumulation regulates which pathways are available (Kohn and Kim, 2015). Focus in biology has often been on the kinetic regulation of fermentation, though recent studies have shown that thermodynamic regulation of rumen fermentation is also important (Kohn, 2014). For example, in the present study, products like CO_2 and acetate could affect the thermodynamic feasibility of reaction pathways producing these products in the fermentation system. When flooding the system with sodium acetate, we are perturbing the in vitro system away from thermodynamic control (equilibrium), therefore allowing us to evaluate the kinetics for the return to equilibrium.

Summary

This study evaluated the effect of starting conditions on VFA and gas production in vitro. We looked at the effects of different ratios of medium:inoculum, substrate (Timothy hay) concentrations, with or without 50 mM sodium acetate addition. This study found that differing ratios of medium:inoculum affect gas production and VFA profile. Higher ratios of medium:inoculum produced more gas. The higher concentration of substrate also produced more gas and increased acetate and propionate production. Most surprising was that the addition of sodium acetate did not affect gas or VFA production. To effectively develop a method to measure VFA and gases, future studies need to further elucidate the in vitro system environment. Other factors that can potentially affect VFA and gas production may include headspace gas composition.

Table 2.1. Main effect of medium:inoculum on gas and VFA production, and pH over 48 hours

	Treatment					
	Medium, %[1]					
Gas Production	**5**	**25**	**50**	**75**	**SEM**	***P***
Total Gas (μmol)[2]	249[c]	357[b]	437[a]	438[a]	12.9	< 0.01
Total Gas/hay (μmol/g)[3]	3418[c]	4863[b]	5944[a]	5825[a]	207.8	< 0.01
Final Gas (μmol)[4]	31[c]	62[b]	90[a]	77[ab]	6.1	< 0.01
Fractional Non-CO_2 (μmol/μmol)[5]	0.41	0.23	0.31	0.23	0.07	0.36
Total VFA Production and pH						
Acetate (mM)	14[b]	20[ab]	22[a]	17[ab]	1.0	< 0.05
Propionate (mM)	5.6	7.7	8.9	7.3	0.84	0.07
Butyrate (mM)	6.0	7.0	4.9	12.0	6.70	0.39
Total VFA (mM)	28	39	44	42	5.3	0.14
Acetate/Propionate[6] (mM/mM)	2.5	2.6	2.6	2.3	0.13	0.19
Acetate/Butyrate (mM/mM)	4.9	5.9	6.6	4.8	0.70	0.22
pH at 48 Hours	5.6[d]	5.9[c]	6.3[b]	6.6[a]	0.01	< 0.01

[a-d] Within a row, means without a common superscript differ (P < 0.05)

[1]Buffer values are reported as percentage of buffer by volume in relation to rumen fluid; 5: 5% buffer 95% rumen fluid, 25: 25% buffer 75% rumen fluid, 50: 50% buffer 50% rumen fluid, 75: 75% buffer 25% rumen fluid

[2]Total Gas is the gas produced between 0 and 48 hours

[3]Total gas/hay was calculated as total gas produced divided by substrate (0.05 or 0.10 g of hay)

[4]Final gas is the gas produced between 24 and 48 h

[5] Non-CO_2 is a fractional value of non-CO_2 gas divided by total gas produced

[6]Initial Rumen Fluid Acetate: Propionate Ratio = 3.7

Table 2.2. Effect of medium:inoculum on the production of gas and VFAs by time[1]

	Treatment					
	Medium, (%)[2]					
	5	**25**	**50**	**75**	**SEM**	***P***
Gas (μmol)						
Initial Concentration (T = 0)	0	0	0	0	--	--
0-4 h	107[a]	113[a]	90[a]	36[b]	8.2	< 0. 01
4-16 h	104[d]	155[c]	222[b]	282[a]	8.4	< 0. 01
16-24 h	7[c]	27[b]	37[ab]	42[a]	3.9	< 0. 01
24-48 h	31[c]	62[b]	90[a]	77[ab]	6.1	< 0. 01
Acetate (mM)						
Initial Concentration (T = 0)	52[a]	45[b]	36[c]	28[d]	1.1	< 0. 01
0-4 h	3.9	4.7	3.8	2.4	0.69	0.14
4-16 h	3.8[b]	7.4[ab]	9.6[a]	8.5[a]	0.95	< 0. 01
16-24 h	3.2	2.2	2.6	4.1	1.30	0.74
24-48 h	4.0[ab]	5.8[ab]	6.4[a]	1.8[b]	1.07	< 0.05
Propionate (mM)						
Initial Concentration (T = 0)	10.3[a]	8.4[b]	5.9[c]	4.1[d]	0.4	< 0. 01
0-4 h	1.4	1.7	1.6	1.1	0.17	0.06
4-16 h	1.1[b]	2.3[a]	3.4[a]	3.2[a]	0.31	< 0. 01
16-24 h	1.1	1.1	1.4	1.4	0.33	0.87
24-48 h	1.8[a]	2.5[a]	2.5[a]	1.6[a]	0.27	< 0.05
Butyrate (mM)						
Initial Concentration (T = 0)	12.3	10.3	4.1	4.8	3.17	0.20
0-4 h	2.4	2.0	0.8	1.3	0.75	0.48
4-16 h	1.5	2.8	2.2	7.6	1.70	0.06
16-24 h	0.8	0.5	0.7	1.5	0.48	0.48

24-48 h	1.7	1.7	1.1	1.8	0.59	0.85
Total VFA (mM)						
Initial Concentration (T = 0)	80[a]	68[a]	49[b]	40[b]	3.8	< 0. 01
0-4 h	8.1	8.9	7.5	4.6	1.34	0.13
4-16 h	7[b]	15[ab]	20[a]	24[a]	2.7	< 0. 01
16-24 h	5.5	4.0	5.0	8.2	2.02	0.52
24-48 h	8	11	11	6	1.7	0.09
Acetate/Propionate (mM/mM)[3]						
0-4 h	2.0	2.2	2.4	2.1	0.52	0.97
4-16 h	3.3	2.8	2.9	2.7	0.28	0.40
16-24 h	2.9	1.7	1.9	2.7	0.80	0.66
24-48 h	2.1	1.5	2.6	4.5	1.11	0.26
Acetate/Butyrate (mM/mM)						
0-4 h	4.2	5.3	6.9	6.0	1.07	0.35
4-16 h	5.7	5.9	6.3	4.1	0.90	0.30
16-24 h	6.6	8.7	6.0	9.2	2.34	0.72
24-48 h	5.0	5.5	6.9	6.5	0.93	0.47

[a-d] Within a row, means without a common superscript differ ($P < 0.05$)

[1]VFA production is calculated as the change is concentration between each interval

[2]Buffer values are reported as percentage of buffer by volume in relation to rumen fluid; 5: 5% buffer 95% rumen fluid, 25: 25% buffer 75% rumen fluid, 50: 50% buffer 50% rumen fluid, 75: 75% buffer 25% rumen fluid

[3]Initial Rumen Fluid Acetate: Propionate Ratio = 3.7

Table 2.3. Main effects of hay on gas and VFA production, and pH over 48 hours

	Treatment			
	Hay (g)[1]			
Gas Production	**0.05**	**0.1**	**SEM**	***P***
Total Gas (μmol)[2]	262[b]	478[a]	9.1	< 0.01
Total Gas/hay (μmol/g)[3]	5244[a]	4781[b]	146.9	< 0.05
Final Gas (μmol)[4]	34[b]	96[a]	4.3	< 0.01
Fractional Non-CO_2 (μmol/μmol)[5]	0.28	0.31	0.05	0.66
Total VFA Production and pH				
Acetate (mM)	14.6[b]	21.9[a]	1.53	< 0.01
Propionate (mM)	5.7[b]	9.1[a]	0.59	< 0.01
Butyrate (mM)	4	4.1	1.21	0.95
Total VFA (mM)	18	22	1.96	0.13
Acetate/Propionate (mM/mM)[6]	2.6	2.4	0.09	0.17
Acetate/Butyrate (mM/mM)	5.0	6.1	0.47	0.10
pH at 48 Hours	6.2[a]	6.0[b]	0.01	< 0.01

[a,b] Within a row, means without a common superscript differ (P < 0.05)

[1]Buffer values are reported as percentage of buffer by volume in relation to rumen fluid; 5: 5% buffer 95% rumen fluid, 25: 25% buffer 75% rumen fluid, 50: 50% buffer 50% rumen fluid, 75: 75% buffer 25% rumen fluid

[2]Total Gas is the gas produced between 0 and 48 hours

[3]Total gas/hay was calculated as total gas produced divided by substrate (0.05 or 0.10 g of hay)

[4]Final gas is the gas produced between 24 and 48 h

[5] Non-CO_2 is a fractional value of non-CO_2 gas divided by total gas produced

[6]Initial Rumen Fluid Acetate: Propionate Ratio = 3.7

Table 2.4. Effect of hay on the production of gas and VFAs by time[1]

	Treatment			
	Hay (g)[2]			
	0.05	**0.1**	**SEM**	***P*-value**
Gas (μmol)				
Initial Concentration (T = 0)	0	0	--	--
0-4 h	65[b]	108[a]	6	< 0.01
4-16 h	149[b]	233[a]	6	< 0.01
16-24 h	14[b]	43[a]	2.8	< 0.01
24-48 h	34[b]	96[a]	4.3	< 0.01
Acetate (mM)				
Initial Concentration (T = 0)	48[b]	51[a]	0.56	< 0.01
0-4 h	3.0[b]	4.4[a]	0.49	< 0.05
4-16 h	6.3[b]	8.3[a]	0.69	< 0.05
16-24 h	2.7	3.3	0.90	0.65
24-48 h	3.0[b]	6.0[a]	0.74	< 0.01
Propionate (mM)				
Initial Concentration (T = 0)	10[b]	12[a]	0.13	< 0.01
0-4 h	1.1[b]	1.8[a]	0.12	< 0.01
4-16 h	2.0[b]	3.1[a]	0.22	< 0.01
16-24 h	1.0	1.5	0.23	0.15
24-48 h	1.5[b]	2.8[a]	0.19	< 0.01
Butyrate (mM)				
Initial Concentration (T = 0)	14	10	3.2	0.49
0-4 h	1.6	1.6	0.53	0.94
4-16 h	3.2	3.9	1.20	0.71
16-24 h	1.3	0.5	0.34	0.08
24-48 h	1.3	1.8	0.42	0.39
Total VFA(mM)				
Initial Concentration (T = 0)	78	79	3.5	0.87
0-4 h	6.5	8.0	0.95	0.26
4-16 h	15	18	1.9	0.21
16-24 h	5.7	5.6	1.39	0.96
24-48 h	6.8[b]	11.5[a]	1.17	< 0.01

Acetate/Propionate (mM/mM)[2]				
0-4 h	2.0	2.4	0.37	0.52
4-16 h	3.3[a]	2.6[b]	0.20	< 0.05
16-24 h	3.1[a]	1.4[b]	0.58	< 0.05
24-48 h	3.3	2.1	0.77	0.26
Acetate/Butyrate (mM/mM)				
0-4 h	4.8	6.5	0.76	0.12
4-16 h	5.5	5.5	0.65	0.95
16-24 h	6.3	8.9	1.70	0.31
24-48 h	6.2	5.8	0.65	0.67

[a,b] Values within a row with different superscripts are statistically different ($P < 0.05$)
[1]VFA production is calculated as the change is concentration between each interval
[2]Hay values represent the levels of substrate used; 0.5 g or 0.10 g of Timothy hay
[3]Initial Rumen Fluid Acetate: Propionate Ratio = 3.7

Table 2.5. Main effect of sodium acetate on gas production, VFA production, and pH

	Treatment			
	Acetate (mM)[1]			
Gas Production	**No**	**Yes**	**SEM**	***P***
Total Gas (μmol)[2]	375	366	9.1	0.49
Total Gas/hay (μmol/g)[3]	5120	4905	146.9	0.3
Final Gas (μmol)[4]	58[b]	72[a]	4.3	< 0.05
Fractional Non-CO_2 (μmol/μmol)[5]	0.35	0.23	0.05	0.12
Total VFA Production and pH				
Acetate (mM)	18.3	18.2	1.53	0.94
Propionate (mM)	7.3	7.4	0.59	0.90
Butyrate (mM)	7.1	7.9	2.24	0.81
Total VFA (mM)	39	38	3.8	0.91
Acetate/Propionate (mM/mM)[6]	2.6	2.4	0.09	0.36
Acetate/Butyrate (mM/mM)	6.1	5.1	0.47	0.14
pH at 48 Hours	6.08[b]	6.13[a]	0.01	< 0.01

[a,b] Within a row, means without a common superscript differ (P < 0.05)
[1]Buffer values are reported as percentage of buffer by volume in relation to rumen fluid; 5: 5% buffer 95% rumen fluid, 25: 25% buffer 75% rumen fluid, 50: 50% buffer 50% rumen fluid, 75: 75% buffer 25% rumen fluid
[2]Total Gas is the gas produced between 0 and 48 hours
[3]Total gas/hay was calculated as total gas produced divided by substrate (0.05 or 0.10 g of hay)
[4]Final gas is the gas produced between 24 and 48 h
[5] Non-CO_2 is a fractional value of non-CO_2 gas divided by total gas produced
[6]Initial Rumen Fluid Acetate: Propionate Ratio = 3.7

Table 2.6. Effect of acetate on the production of gas and VFAs by time[1]

	Treatment			
	Acetate (mM)[1]			
	N	Y	SEM	*P*
Gas (mmol)				
Initial Concentration (T = 0)	0	0	--	--
0-4 h	103[a]	70[b]	6	< 0.05
4-16 h	190	192	6	0.89
16-24 h	24[b]	33[a]	2.8	< 0.05
24-48 h	58[b]	72[a]	4.3	< 0.05
Acetate (mM)				
Initial Concentration (T = 0)	36[b]	63[a]	0.6	< 0.01
0-4 h	3.5	3.9	0.49	0.52
4-16 h	7.0	7.6	0.70	0.51
16-24 h	2.5	3.5	0.90	0.46
24-48 h	5.4	3.7	0.74	0.10
Propionate (mM)				
Initial Concentration (T = 0)	10.9	10.6	0.13	0.07
0-4 h	1.5	1.4	0.12	0.70
4-16 h	2.4	2.6	0.22	0.49
16-24 h	1.1	1.4	0.23	0.27
24-48 h	2.2	2.1	0.19	0.63
Butyrate (mM)				
Initial Concentration (T = 0)	11	13	3.2	0.76
0-4 h	1.7	1.5	0.53	0.83
4-16 h	3.3	3.8	1.23	0.76
16-24 h	0.6	1.2	0.34	0.23
24-48 h	1.6	1.5	0.43	0.84
Total VFA(mM)				
Initial Concentration (T = 0)	65[b]	92[a]	3.5	< 0.01
0-4 h	7.2	7.3	0.95	0.91
4-16 h	16	17	1.9	0.87
16-24 h	4.7	6.6	1.39	0.32
24-48 h	10	8	1.2	0.16
Acetate/Propionate (mM/mM)[2]				
0-4 h	2.3	2.1	0.37	0.70
4-16 h	3.0	2.9	0.20	0.68
16-24 h	2.6	2.0	0.58	0.47
24-48 h	2.5	2.9	0.77	0.71

Acetate/Butyrate (mM/mM)				
0-4 h	5.7	5.5	0.76	0.86
4-16 h	6.1	4.9	0.60	0.17
16-24 h	8.5	6.7	1.70	0.46
24-48 h	6.3	5.6	0.66	0.45

[a,b] Values within a row with different superscripts are statistically different ($P < 0.05$)

[1]VFA production is calculated as the change is concentration between each interval

[2]Acetate treatment indicates the addition of sodium acetate (NaOAc); N: no acetate, Y: 50 mM NaOAc

[2]Initial Rumen Fluid Acetate: Propionate Ratio = 3.7

Figure 2.1. The effect of increasing ratio of medium:inoculum (% by volume) and sodium acetate addition (50 mM NaOAc) on gas production (μmol) between 24 and 48-h. Medium:inoculum values are reported as percentage of sodium bicarbonate buffered medium by volume in relation to rumen fluid: 5% medium and 95% rumen fluid, 25% medium and 75% rumen fluid, 50% medium and 50% rumen fluid, and 75% medium and 25% rumen fluid. Gas production increased as medium increased with acetate and averaged (29, 77, 104, 76 μmol) with acetate vs. (33, 46, 76, 76 μmol) without acetate; SE = ± 10.2 μmol. Significance was determined at $P < 0.05$ and a trend at $P < 0.10$. Multiple mean comparisons test was conducted using Tukey's adjustment. Values are reported as the mean ± S.E. and means with different letters (a, b, c, d) are significantly different.

Figure 2.1.

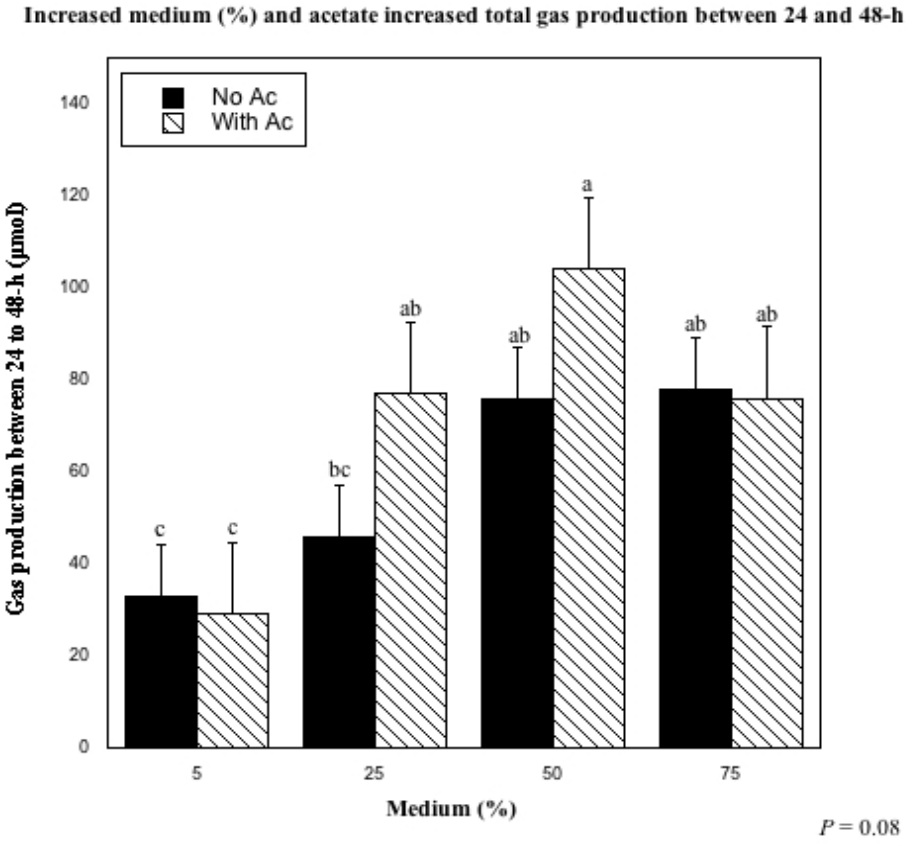
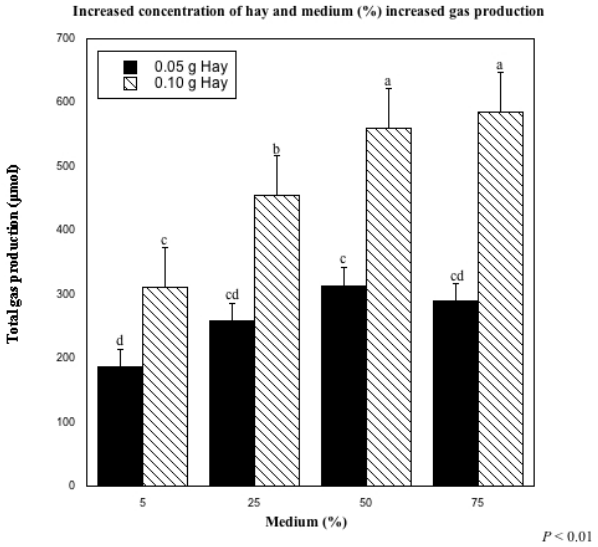

Increased medium (%) and acetate increased total gas production between 24 and 48-h
No Ac
With Ac
Gas production between 24 to 48-h (µmol)
140
120
100
80
60
40
20
0
c
c
bc
ab
ab
a
ab
ab
5
25
50
75
Medium (%)
P = 0.08

Figure 2.2. The effect of increasing ratio of medium:inoculum (% by volume) and Timothy hay (g) on: A) total gas production (186, 258, 314, and 290 μmol average per treatment) for lower hay concentration versus (312, 456, 560, and 585 μmol average per treatment) for higher hay concentration; SE = ± 21.0; and B) gas production (μmol) between 24 and 48-h (10, 34, 46, and 46 μmol average per treatment) for lower hay concentration versus (53, 89, 133, and 108 μmol average per treatment) for higher hay concentration, SE = ± 10.2 μmol. Significance was determined at $P < 0.05$ and a trend at $P < 0.10$. Multiple mean comparisons test was conducted using Tukey's adjustment. Values are reported as the mean ± S.E. and means with different letters (a, b, c, d) are significantly different.

Figure 2.2.


Increased concentration of hay and medium (%) increased gas production
0.05 g Hay
0.10 g Hay
Total gas production (µmol)
Medium (%)
P < 0.01

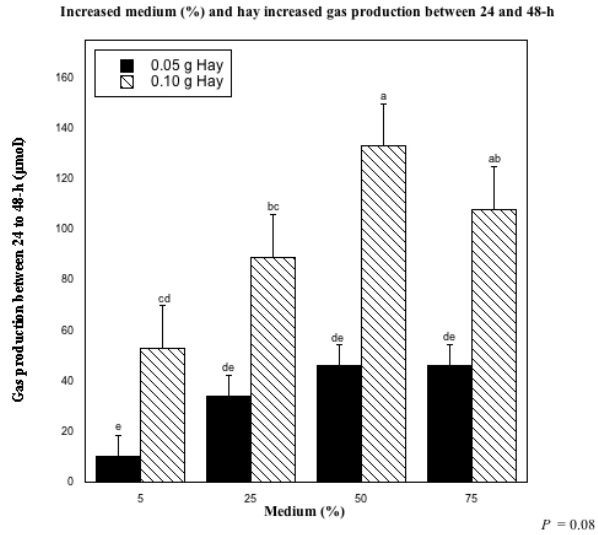
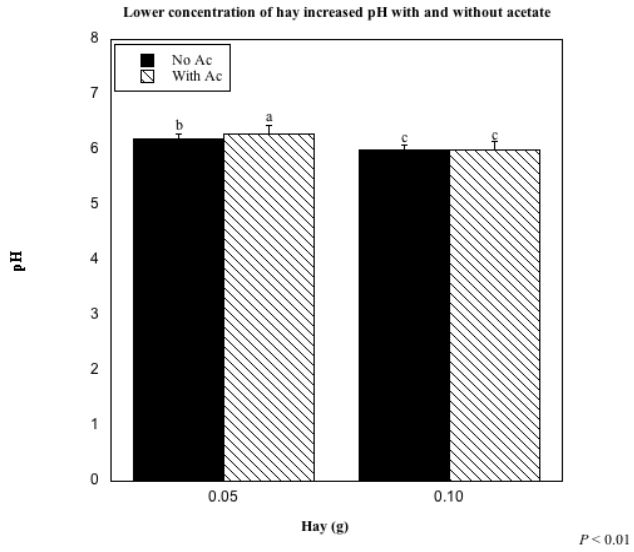
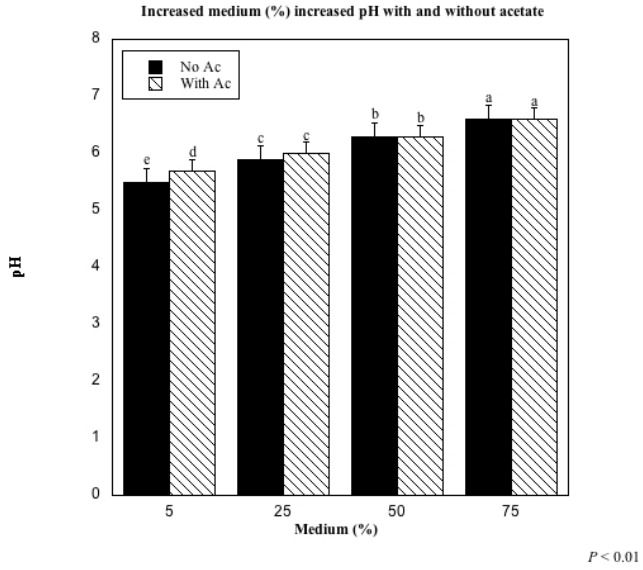
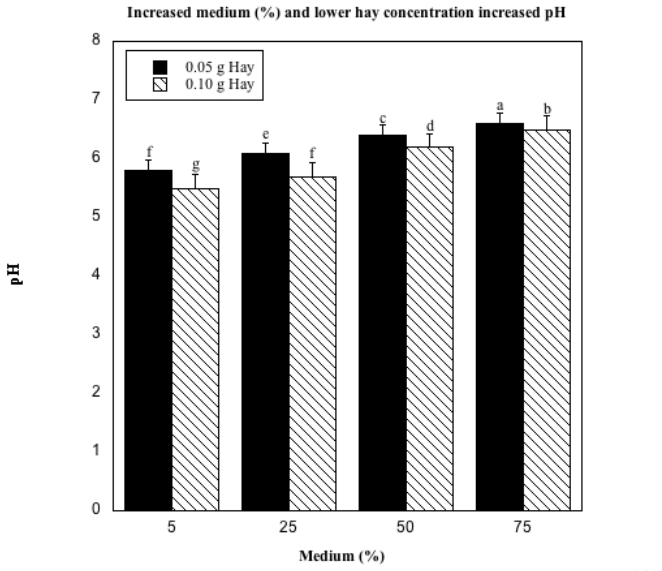

Increased medium (%) and hay increased gas production between 24 and 48-h
0.05 g Hay
0.10 g Hay
Gas production between 24 to 48 h (µmol)
Medium (%)
P = 0.08

Figure 2.3. The effect of A) sodium acetate addition (NaOAc, 50mM) and hay on pH (6.3 vs 6.0) with acetate and (6.2 vs 6.0) without acetate, SE = ± 0.01; B) ratio of medium:inoculum and acetate addition on pH (5.7, 6.0, 6.3 and 6.6) with acetate vs (5.5, 5.9, 6.3, and 6.6) without acetate, SE = ± 0.02; and C) ratio of medium:inoculum and hay (5.8, 6.1, 6.4, and 6.6) for lower concentration of hay vs (5.5, 5.7, 6.2, and 6.5) for higher hay concentration, SE = ± 0.02. Significance was determined at $P < 0.05$ and multiple mean comparisons test was conducted using Tukey's adjustment. Values are reported as the mean ± S.E. and means with different letters (a, b, c, d) are significantly different.

Figure 2.3.


Lower concentration of hay increased pH with and without acetate
No Ac
With Ac
pH
Hay (g)
0.05
0.10
P < 0.01


Increased medium (%) increased pH with and without acetate
No Ac
With Ac
pH
Medium (%)
5
25
50
75
P < 0.01


Increased medium (%) and lower hay concentration increased pH
0.05 g Hay
0.10 g Hay
pH
Medium (%)
5
25
50
75
f
g
e
f
c
d
a
b
P < 0.01

CHAPTER THREE

EFFECT OF ACETATE ADDITION AND HEADSPACE GAS COMPOSITION ON IN VITRO PRODUCTION OF VOLATILE FATTY ACIDS AND GASES

L. M. JUDD and R. A. KOHN

ABSTRACT: The development of in vitro methods to accurately estimate gas production and volatile fatty acid (VFA) profile in rumen fermentation would enable isolation of fermentation effects from animal interactions. The purpose of this experiment was to examine the effects of different ratios of gases (CO_2, CH_4, and H_2) and acetate addition on VFA profile. Experimental design was a 4 × 2 factorial CRD with 4 replicates. Tubes were subjected to different experimental combinations including 4 different gas profiles, with or without addition of 50 mM sodium acetate. Gas headspace treatments were by volume: 1) CO_2 (100%), 2) CO_2-CH_4 (50/50), 3) CO_2-H_2 (95/5), and 4) CO_2-CH_4-H_2 (47.5/47.5/5). Each treatment was replicated in 4 tubes with repeated measures of VFA and gas volume taken at 0, 4, 16, 24, and 48 hours. Timothy hay (1%) and 0.5 ml sodium acetate solution or buffered medium were added to each 20-ml tube. Tubes were equilibrated with each gas mixture before adding 9.5 mL rumen fluid. Tubes were incubated at 39°C while shaking with 20-ml syringes attached to collect and measure produced gases. There was an effect ($P < 0.01$) of gas composition on gas production between 0 to 4 hours. Lower starting concentration of CO_2 in headspace gas may have caused CO_2 efflux from the buffer. There was a trend ($P < 0.10$) on propionate production by gas composition between 0 and 4 h. Butyrate production between 0 to 4 hours was affected ($P = 0.05$) by gas composition and was lower with lower initial concentration of CO_2. There was a trend ($P < 0.10$) of butyrate production between 4 to 16 hours. There was an effect ($P < 0.05$) of the gas mixture on acetate production from 24 to 48 h and on the acetate:propionate (A:P) ratio of produced VFA. In contrast to expectation, there was a tendency towards added sodium acetate decreasing acetate production ($P < 0.10$) between 0 to 4 h but decreasing

($P < 0.05$) acetate production between 16 to 24 h. Initial gas composition of in vitro procedures can affect gas production and VFA profiles with higher percentage of CH_4 and H_2 in headspace (more reduced conditions) favoring propionate and butyrate over acetate and gas production.

Key words: fermentation gases, in vitro procedures, methane, rumen fermentation, sodium acetate, volatile fatty acids

INTRODUCTION

There is a need for an improved in vitro method that would enable us to study factors that affect the profile of produced VFA and gases. In vitro methods have been developed to measure digestibility, but such methods may not accurately estimate VFA profile or methane production. Accumulation of products such as certain VFA or gases can cause a thermodynamic shift in production of subsequent products (Ungerfeld and Kohn, 2006). Therefore, which VFA or gases are produced could depend on accumulation of VFA or gases in vitro, but most current in vitro methods use liquid and gas media that have low concentrations of VFA or gases other than CO_2.

In a previous study (Judd and Kohn, 2018), we evaluated the effect of buffering and substrate availability of an in vitro system on VFAs and gas profile. The present study will evaluate the effect of headspace gas composition and acetate concentration on gas production and VFA profile in vitro. We hypothesize that headspace gas composition will affect the VFA profile and acetate: propionate (A/P) production ratio and that the addition of acetate will shift fermentation away from acetate and towards propionate and butyrate due to thermodynamic control.

The purpose of this experiment was to advance development of an in vitro technique that mimics an in vivo rumen environment to study VFA profiles and gas production during fermentation. We examined the effects of headspace gas composition and acetate addition on the VFA profile in a closed in vitro system. The goal was to further research to develop an in vitro method in which VFA and gas profiles are similar to what is observed in vivo. Such a method should help elucidate the mechanisms (kinetics and thermodynamics) behind control of ruminal fermentation. Measurements of VFA, pH, and

gas production were made for differing initial headspace gas mixtures, and with or without sodium acetate addition.

MATERIALS AND METHODS

Experimental procedures were approved by the University of Maryland Institutional Animal Care and Use Committee (IACUC) [398173-1].

Experimental Design and Treatments

The experimental design was a 4 × 2 factorial CRD; the treatments were gas mixture combinations (calculated by volume at ratios of 100% CO_2, 50:50 CH_4-CO_2, 95:5 CO_2-H_2, and 47.5:47.5:5 CH_4-CO_2-H_2), and sodium acetate (50mmol NaOAc addition or not). Each treatment was replicated 4 times. Total volume of each treatment in the in vitro tubes was 10 ml per 20-ml Hungate tube. A previous study (Judd and Kohn, 2018) utilizing different substrate amounts did not show a significant difference of substrate effect on treatment, therefore 0.10 g of Timothy hay was used in this experiment. Rumen fluid at a concentration of 100% was used in this experiment to focus on the effect of gas composition and acetate.

Rumen Fluid Collection and Sample Preparation.

Rumen fluid was collected according to the IACUC protocol from a permanently non-lactating rumen-cannulated cow consuming a timothy hay diet. Approximately 0.5 -1 L of rumen fluid (solids and liquid) was collected anaerobically in 50-mL centrifuge tubes. Rumen fluid was infused with CO_2 and blended for 20 seconds. Rumen fluid was strained through 4 layers of cheesecloth and glass wool into a 1-L flask infused with CO_2. The strained rumen fluid was infused with CO_2 and stirred continuously with a magnetic bar.

Timothy hay was pre-weighed (0.10 g) into labeled 20-mL Hungate glass tubes with a rubber stopper and screw caps. The Timothy hay was measured on a Mettler Toledo AE260 Delta Range (Columbus, OH) 4-place balance. The average weight for the 0.10 g Timothy hay was 0.107 g (SD = 0.002). The in vitro medium buffered medium was prepared, perfused with CO_2, and reduced with a reducing agent as previously published (Goering and Van Soest, 1970). The Hungate tubes were sealed with rubber stoppers and screw caps. Air was removed from each tube using a Precision vacuum pump (Precision Scientific, Chicago, IL) to 0.5 atm. In each 20-mL tube, 20 ml of the gas mixture treatment was added in random order using a 20-mL gas-tight syringe fitted with a 27-gauge needle and 3-way stopcock (Cole-Parmer, Vernon Hills, IL). The tube was vacuumed, then another 20 ml of the gas mixture added. A third and final vacuum was performed, and 20 ml of the gas treatment was added, and the syringe locked with a 3-way stopcock and remained in the Hungate tube. The process to add the treatments was conducted anaerobically and 9.5 ml of rumen fluid was added to each tube in randomized order. The 20-mL syringes had tick marks indicated intervals of 0.2-mL. Tubes were then given either 0.5 ml of 1M acetate (NaOAc) (this increased starting concentration of acetate for these treatments by 50 mM), or 0.5 ml of in vitro medium by inserting a 3-mL gas-tight syringe with 27-gauge needle into the in vitro tubes containing rumen fluid (9.5 ml) was added in random order using a 10 ml gas-tight syringe attached to a 27-gauge needle to each 20-mL Hungate tubes. Due to the increase in pressure above 1 atm, the 10-mL syringe was not immediately removed during the addition of rumen fluid. The addition of the rumen fluid and NaOAc caused the 20-mL syringe to rise to 10 ml, the 20 mL syringe was then pressed down so the excess air was expelled back into the 10-mL syringe. The 10-mL syringe and

needle were then removed, and the air was expelled. Tubes were subsequently incubated at 39°C.

Gas volume was recorded and liquid in each tube measured before placing the samples in the incubator (39°C) in a dry water bath that was shaking and mixing the tubes. Liquid samples for volatile fatty acids (VFAs) were collected by inverting the in vitro tube, allowing the substrate to settle then using a 27-gauge needle and 5-ml gas-tight syringe to withdraw 1 mL of sample. The sample was expelled into a 1.5 mL microcentrifuge tube and frozen in a -20°C freezer for further analysis. Mean retention times of lactating and dry cows are 20 to 24 hours for grains or 30 hours for forage (Hartnell and Satter, 1979; Ishler et al., 1996), therefore this study used 48 hours as an endpoint for fermentation and measurements of gas production and VFAs were recorded at 0, 4, 16, 24, and 48 hours.

Gas Measurement

Gas production was measured in the incubator at 39°C, recorded in mL and converted to μmol by dividing the average gas produced for each treatment by 25.6 mL/mmol and multiplying by 1000 μmol/mmol as stated by the ideal gas law. Gas was recorded at each timepoint (0, 4, 16, 24, and 48 hours) and expelled from the syringe. The syringe was then screwed back onto the needle.

VFA Analysis

The VFA samples were prepared using a modified method (Erwin et al., 1961). The 1.5 mL microcentrifuge tubes containing the VFA samples were thawed at room temperature then spun in a centrifuge at 12,000 x g for 30 minutes. Phosphoric acid (10% H_3PO_4) was added to the supernatant of each sample. VFA concentrations were then

measured using gas chromatography (GC) (Hewlett-Packard model 6890) with a 4.6 m length x .318 cm outer diameter x 2.1 mm inner diameter packed GC column (60/80 Carboxen-1000 support, model 1-2390, Supelco, Inc, Bellefonte, PA), and flame ionization detector (FID). The split ratio of the injector port (220°C) was 100:1. Helium was used as a carrier gas with a flow of 40 mL/min. The initial column temperature was 130°C held for 10 min, then increased to 200 °C (ramp of 80°C/min) for 1 min, and a post-run temperature of 120°C. The detector temperature was 200°C with a hydrogen and air flow of 40 mL/min and 200 mL/min, respectively. VFA production is reported as the change in concentration at each interval.

Statistical Analysis

Statistical analyses were conducted using JMP Pro 12 (JMP®, Version *12*. SAS Institute Inc., Cary, NC, 1989-2007). There were two mixed models used. The first model was: $Y_{ijklm} = \mu+G_i+A_j+T_k+\gamma_{ijk(l)}+\varepsilon_{ijkl(m)}$ for response variables measured over time within tubes. The second model was: $Y_{ijkl} = \mu+G_i+A_j+\gamma_{ij(k)}+\varepsilon_{ijk(l)}$ for response variables measured only once. For each model Y is the response, μ is the mean of the population, G_i is the effect of gas mix of 100% CO_2, 50:50 CH_4-CO_2, 95:5 CO_2-H_2, and 47.5:47.5:5 CH_4-CO_2-H_2, A_j is the effect of acetate, with or without 50 mM addition, T_k is time measured at 0, 4, 16, 24, and 48 h, $\gamma_{ijk(l)}$ is the random effect of the tube nested in treatment (gas and acetate combinations or gas acetate and time), and $\varepsilon_{ijkl(m)}$ is the residual effect. All interactions were included in each model and time was a continuous variable. This model measured the effect of treatment on total gas production, pH, and VFA production over time.

RESULTS AND DISCUSSION

Effect of Headspace Gas Composition

Table 3.1 shows the main effects of gas mixture treatments on cumulative gas and VFA production. There was no effect of initial gas mixture on total gas or VFA production. We expected that total propionate production might be higher in more reduced treatments (CH_4 or H_2). The gas treatments that had equal proportions of CO_2 and CH_4 (50/50 CO_2-CH_4 and 47.5/47.5/5 CO_2-CH_4-H_2) may have inhibited the synthesis of methane, thereby leaving more hydrogen to be readily available for the synthesis of propionate or by shifting fermentation away from acetate towards butyrate. The presence of hydrogen in the 47.5/47.5/5 CO_2-CH_4-H_2 gas mixture could increase propionate production as an alternative to increased hydrogen formation by utilizing the hydrogen present to synthesize propionate (Janssen, 2010). Lower pH in a system can increase propionate production by inhibiting methanogens, and decreasing competition with methane synthesis for the available hydrogen resource (Russell, 1998).

Table 3.2 shows there was an effect ($P < 0.01$) on gas production between 0 and 4 h of the gas mixture treatments. Gas production was highest when the initial composition contained methane (50/50 CO_2-CH_4). Studies have shown that initial gas composition of in vitro procedures can affect gas production (Menke et al., 1979; Jensen and Jørgensen, 1994; Getachew et al., 1998). Gas production is affected by fermentation and the presence of a bicarbonate buffer (Getachew et al., 1998). Lower starting concentration of CO_2 in the gas headspace may have increased CO_2 efflux from the bicarbonate buffer.

Volatile fatty acid production was affected by the gas mixtures at different time

intervals. There was a trend ($P < 0.10$) for initial gas composition to affect propionate production between 0 to 4 h and propionate production was lower in the treatments with hydrogen (Table 3.2). The presence of higher concentrations of H_2 in the headspace with CO_2 may have shifted fermentation away from the production of acetate which produces H_2, and toward propionate which uses H_2 (Kohn and Kim, 2015). Butyrate production was affected ($P = 0.05$) by initial gas composition between 0 to 4 h (Table 3.2). Butyrate decreased as hydrogen increased in the gas mixtures. There was a trend ($P < 0.10$) for gas to affect butyrate production between 4 to 16 h with higher butyrate with increased hydrogen. This is the opposite effect observed in the previous time interval. One study (El-Gammal et al., 2017), observed a similar increase in butyrate in the presence of pure CO. There was an effect ($P < 0.05$) of gas mixture on the A: P production ratio between 24 and 48 h. The 95/5 CO_2-H_2 had the highest A: P production and the 50/50 CO_2-CH_4 mixture had the lowest. There was no effect of headspace gas treatment on the production of acetate, total VFA, or A: B ratio by time.

Effect of Acetate

Sodium acetate addition (50 mM NaOAc) has been used in previous studies (Judd and Kohn, 2018) to test the hypothesis that the addition of acetate into a system will affect fermentation and the VFA and gas profiles by shifting production of VFAs from acetate to propionate or butyrate. There was an effect (Table 3.3) of acetate treatment on pH at 48 h and pH was highest ($P < 0.05$) for the treatments with NaOAc addition. The average pH of the samples with and without acetate addition was 5.8 and 5.7, respectively. As the pH declines, the additional acetate would accept a proton and act as a buffer. There was a trend ($P < 0.10$) towards increased production of acetate with acetate treatment between 0 and 4

h (Table 3.4). The increased acetate production is contrary to our hypothesis. Acetate addition decreased ($P < 0.05$) acetate production between 16 and 24 h. Total VFA production (Table 3.4) tended to be lower ($P < 0.10$) between 16 to 24 h for the acetate treatment. Due to the difference in acetate production between 0 and 4 h the A: P and A: B ratio of produced acids were higher ($P < 0.05$) for the acetate treatment. There was no effect of acetate on production of gas, propionate, or butyrate.

Effect of Treatment Interactions

There was an effect ($P = 0.05$) of gas mixture and acetate addition on the acetate:propionate production ratio 0 to 48 hours (Fig.3.1). With the exception of the 100% CO_2 treatment the acetate:propionate production ratio was lower for the gas mixture treatments with acetate addition. There was a trend ($P < 0.10$) of gas mixture and acetate addition on the acetate:propionate production ration between 4 to 16 h (Fig. 3.2). The treatments that were more reduced had higher acetate:propionate ratios with acetate addition. During this time interval the 100% CO_2 and 95/5 CO_2-H_2 had lower acetate:propionate ratios with acetate addition. Gas production parameters were not affected by the gas mixture by acetate addition interaction. There was no effect of the gas mixture by acetate addition interaction on the individual production of VFAs from 0 to 48-h (cumulative production) or by time. There is a direct correlation of acetate to propionate ratio from fermentation in the rumen with the dietary the forage: concentrate (F:C) ratio (Moss et al., 2000). This study only evaluated the effects of Timothy hay as a substrate. It is possible that in the presence of a different substrate such as grain there would have been more utilization of the hydrogen that was present in these treatments and potentially lower acetate:propionate ratios due to a shift in VFA profiles.

Summary

This experiment evaluated the role of headspace gas on VFA and gas profiles. VFA profiles with a higher percentage of CH_4 and H_2 in the headspace favored propionate and butyrate over acetate and gas production. The addition of sodium acetate did not decrease the production of acetate as hypothesized, however the feed source may have also played a role in favoring the production of acetate over propionate or butyrate. Future studies will further evaluate the role of sodium acetate addition in an in vitro system. A higher concentration of sodium acetate may be more effective in shifting fermentation away from acetate and towards propionate or butyrate.

We examined the effects of headspace gas composition and acetate addition on the VFA profile in a closed in vitro system. The goals of this study are to further develop an in vitro model that can potentially be comparable to an in vivo model. The development of such a model will aid in a better understanding of the mechanisms (kinetics and thermodynamics) behind rumen fermentation. The in vitro tube in this study was subjected to experimental combinations of differing headspace gas mixtures, sodium acetate addition, and measurements of VFAs, pH, and gas production.

ACKNOWLEDGEMENTS

The authors thank Rachel Rha and Jillian Yant for their assistance in conducting this study.

Table 3.1. Main effect of gas mixture on gas and VFA production, and pH over 48 hours

	Treatment					
	Gas Mix, %[1]					
Gas Production	**CO_2**	**CO_2-CH_4**	**CO_2-H_2**	**CO_2-CH_4-H_2**	**SEM**	***P***
Total Gas (μmol)[2]	182	304	225	224	38.7	0.16
VFA Production and pH						
Acetate (mM)	22	22	19	21	2.4	0.77
Propionate (mM)	8.7	9.0	7.1	9.7	0.78	0.15
Butyrate (mM)	8.1	8.6	7.4	9.4	0.74	0.34
Total VFA (mM)	41	43	37	45	3.9	0.52
Acetate/Propionate[3] (mM/mM)	2.5	2.4	2.6	2.1	0.18	0.41
Acetate/Butyrate (mM/mM)	2.6	2.5	2.5	2.2	0.23	0.70
pH at 48 Hours[4]	5.8	5.7	5.8	5.7	0.05	0.17

[a-b] Values within a row with different superscripts are statistically different ($P < 0.05$)

[1] Gas mix is the different gas combinations (% by volume); CO_2: 100% CO_2, CO_2-CH_4: 50% CO_2- 50% CH_4, CO_2-H_2: 95% CO_2- 5% H_2, CO_2-CH_4-H_2: 47.5% CO_2- 47.5% CH_4- 5%H_2

[2]Total Gas is the gas produced between 0 and 48 hours

[3] Initial Rumen Fluid Acetate: Propionate Ratio = 3.65

[4] Initial pH = 6.61

Table 3.2. Effect of gas mix on the production of gas and VFAs by time[1]

	Treatment					
	Gas Mix, (%)[2]					
	CO_2	CO_2-CH_4	CO_2-H_2	CO_2-CH_4-H_2	SEM	*P*
Gas (μmol)						
Initial Concentration (T = 0)	0	0	0	0	--	--
0-4 h	42[b]	105[a]	38[b]	56[b]	12.5	< 0. 01
4-16 h	92	149	137	104	25.6	0.39
16-24 h	7	20	34	30	9.6	0.21
24-48 h	41	35	17	20	12.7	0.45
Acetate (mM)						
Initial Concentration (T = 0)	70	73	68	68	2.3	0.40
0-4 h	10.7	9.8	8.7	6.5	1.96	0.47
4-16 h	6.3	8.5	7.1	6.6	1.06	0.53
16-24 h	0.7	-0.2	2.0	1.4	0.99	0.45
24-48 h	3.9	4.2	0.9	6.5	1.73	0.21
Propionate (mM)						
Initial Concentration (T = 0)	13.9	14.3	14.2	14.1	0.30	0.88
0-4 h	2.2	2.5	1.8	1.3	0.34	0.08
4-16 h	2.8	3.4	3.4	3.2	0.47	0.77
16-24 h	0.7	0.5	0.8	1.1	0.32	0.66
24-48 h	3.0	2.9	1.1	3.7	0.90	0.23
Butyrate (mM)						
Initial Concentration (T = 0)	8.2	8.4	8.3	8.2	0.33	0.95
0-4 h	3.0	3.1	2.3	1.4	0.44	0.05
4-16 h	2.5	3.1	3.5	4.4	0.52	0.08
16-24 h	0.4	0.3	0.5	0.8	0.26	0.51
24-48 h	2.3	2.3	1.1	2.4	0.57	0.33
Total VFA (mM)						
Initial Concentration (T = 0)	90	94	89	88	2.9	0.53
0-4 h	18	17	14	10	2.8	0.23
4-16 h	12	16	15	16	1.9	0.57
16-24 h	1.8	0.7	3.7	3.8	1.65	0.48
24-48 h	9.6	9.9	3.7	14.2	3.40	0.24
Acetate/Propionate (mM/mM)[3]						
0-4 h	4.6	4.0	4.9	4.9	0.35	0.28

4-16 h	2.5	2.8	2.1	2.1	0.30	0.30
16-24 h	1.4	0.8	0.5	4.1	1.59	0.38
24-48 h	1.9[ab]	0.2[b]	3.5[a]	1.4[ab]	0.85	0.05
Acetate/Butyrate (mM/mM)						
0-4 h	3.3	3.2	4.5	4.3	0.55	0.30
4-16 h	2.6	2.7	2.3	1.8	0.30	0.17
16-24 h	4.0	1.0	7.3	4.5	2.50	0.39
24-48 h	1.7	1.2	3.1	2.4	0.65	0.20

[a-b] Values within a row with different superscripts are statistically different ($P < 0.05$)
[1]VFA production is calculated as the change is concentration between each interval
[2]Gas mix is the different gas combinations (% by volume); CO_2: 100% CO_2, CO_2-CH_4: 50% CO_2- 50% CH_4, CO_2-H_2: 95% CO_2- 5% H_2, CO_2-CH_4-H_2: 47.5% CO_2- 47.5% CH_4- 5%H_2

[3] Initial Rumen Fluid Acetate: Propionate Ratio = 3.65

Table 3.3. Main effect of sodium acetate on gas production, VFA production, and pH[1]

	Treatment			
	Acetate (mM)[1]			
Gas Production	**No**	**Yes**	**SEM**	***P***
Total Gas (μmol)[2]	264	203	27.5	0.14
Total VFA Production and pH				
Acetate (mM)	20	22	1.7	0.46
Propionate (mM)	8.4	8.9	0.55	0.55
Butyrate (mM)	8.2	8.6	0.53	0.57
Total VFA (mM)	40	43	2.7	0.57
Acetate/Propionate (mM/mM)[3]	2.4	2.4	0.13	0.70
Acetate/Butyrate (mM/mM)	2.4	2.5	0.16	0.80
pH at 48 Hours[4]	5.7[b]	5.8[a]	0.04	< 0.05

[a-b]Within a row, means without a common superscript differ ($P < 0.05$)
[1]Acetate treatment indicates the addition of sodium acetate (NaOAc); N: no acetate, Y: 50 mM NaOAc. Initial Rumen Fluid Acetate: Propionate Ratio = 3.65
[2]Total Gas is the gas produced between 0 and 48 hours
[3] Initial Rumen Fluid Acetate: Propionate Ratio = 3.65
[4] Initial pH = 6.61

Table 3.4. Effect of acetate on the production of gas and VFAs by time[1]

	Treatment			
	Acetate (mM)[2]			
	N	Y	SEM	*P*
Gas (μmol)				
Initial Concentration (T = 0)	0	0	--	--
0-4 h	66	54	8.9	0.34
4-16 h	134	106	18.1	0.28
16-24 h	28	18	6.8	0.30
24-48 h	35	21	9.0	0.28
Acetate (mM)				
Initial Concentration (T = 0)	66[b]	105[a]	1.4	< 0.01
0-4 h	7.1	10.7	1.39	0.08
4-16 h	7.7	6.6	0.75	0.31
16-24 h	2.0[a]	-0.1[b]	0.70	< 0.05
24-48 h	3.0	4.8	1.22	0.31
Propionate (mM)				
Initial Concentration (T = 0)	19	19	0.3	0.99
0-4 h	1.7	2.1	0.24	0.27
4-16 h	3.4	3.1	0.33	0.61
16-24 h	1.0	0.6	0.23	0.24
24-48 h	2.3	3.0	0.64	0.49
Butyrate (mM)				
Initial Concentration (T = 0)	14	14	0.3	0.75
0-4 h	2.4	2.5	0.31	0.76
4-16 h	3.3	3.4	0.37	0.79
16-24 h	0.7	0.4	0.18	0.29
24-48 h	1.8	2.2	0.40	0.52
Total VFA (mM)				
Initial Concentration (T = 0)	101[b]	139[a]	1.8	< 0.01
0-4 h	13	17	2.0	0.18
4-16 h	16	14	1.4	0.46
16-24 h	4.0	1.0	1.2	0.09
24-48 h	7.9	10.8	2.41	0.41
Acetate/Propionate (mM/mM)[3]				
0-4 h	4.2[b]	5.0[a]	0.25	< 0.05
4-16 h	2.4	2.3	0.21	0.87

16-24 h	2.0	1.4	1.12	0.70
24-48 h	1.2	2.3	0.60	0.23
Acetate/Butyrate (mM/mM)				
0-4 h	3.1[b]	4.5[a]	0.39	< 0.05
4-16 h	2.5	2.2	0.21	0.22
16-24 h	3.9	4.5	1.77	0.80
24-48 h	1.6	2.6	0.46	0.13

[a-b]Within a row, means without a common superscript differ ($P < 0.05$)

[1]VFA production is calculated as the change is concentration between each interval

[2]Acetate treatment indicates the addition of sodium acetate (NaOAc); N: no acetate, Y: 50 mM NaOAc. Initial Rumen Fluid Acetate: Propionate Ratio = 3.65

[3] Initial Rumen Fluid Acetate: Propionate Ratio = 3.65

Figure 3.1. The effect of gas composition (% by volume) and sodium acetate addition (50 mM NaOAc) on cumulative acetate:propionate production ratio. Headspace gas composition reported as treatment number 1-4: 1) 100% CO_2, 2) 50:50 CO_2-CH_4, 3) 95:5 CO_2-H_2, and 4) 47.5:47.5:5 CO_2-CH_4-H_2. Acetate:propionate ratio was decreased in treatments 2-4 with the addition of sodium acetate. The acetate:propionate ratio averaged 2.6, 2.7, and 2.27 without acetate vs 2.3, 2.5, and 2.1; SE = ± 0.25 mM with acetate for the 50:50 CO_2-CH_4, 95:5 CO_2-H_2, and 47.5:47.5:5 CO_2-CH_4-H_2 treatments, respectively. In the 100% CO_2 treatment acetate:propionate ratio was lower without acetate (2.0) vs with acetate (2.9); SE = ± 0.25 mM. A trend was determined at $P < 0.10$. Values are reported as the mean ± S.E.

Figure 3.1.

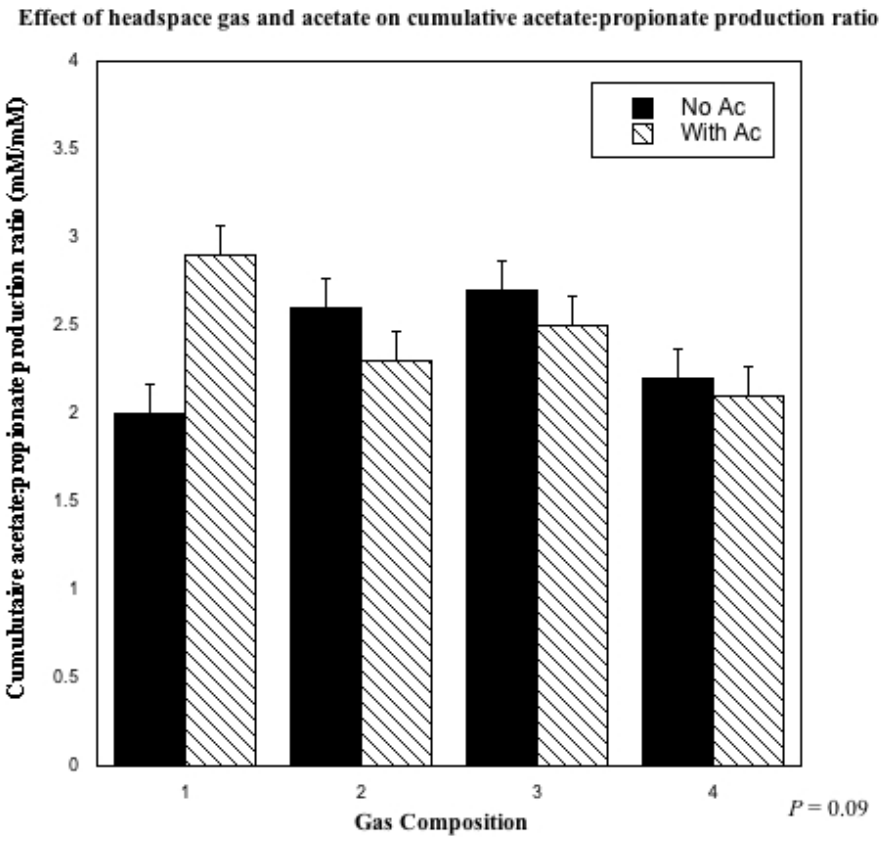
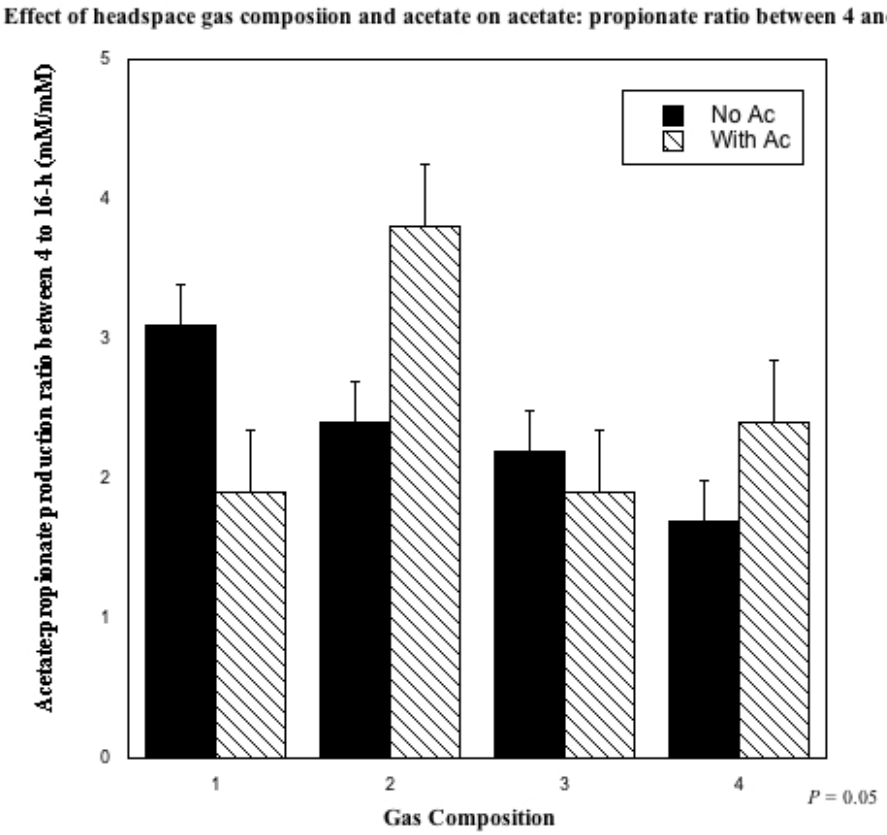

Effect of headspace gas and acetate on cumulative acetate:propionate production ratio
Cumulutaive acetate:propionate production ratio (mM/mM)
4
3.5
3
2.5
2
1.5
1
0.5
0
No Ac
With Ac
1
2
3
4
Gas Composition
P = 0.09

Figure 3.2. The effect of gas composition (% by volume) and sodium acetate addition (50 mM NaOAc) on acetate:propionate production ratio between 4 and 16-h. Headspace gas composition reported as treatment number 1-4: 1) 100% CO_2, 2) 50:50 CO_2-CH_4, 3) 95:5 CO_2-H_2, and 4) 47.5:47.5:5 CO_2-CH_4-H_2. Acetate:propionate ratio was lower in the more reduced treatments (50:50 CO_2-CH_4 and 47.5:47.5:5 CO_2-CH_4-H_2) without acetate and averaged 2.4 and 1.7 compared to with acetate 3.8 and 2.4 respectively; SE = ± 0.47 mM. Conversely, in the 100% CO_2 and 95:5 CO_2-H_2 treatments the acetate:propionate ratio was higher without acetate (3.1 and 2.2) than with acetate treatment (1.9 and 1.9); SE = ± 0.47 mM. A trend was determined at $P < 0.10$. Values are reported as the mean ± S.E.

Figure 3.2.


Effect of headspace gas composiion and acetate on acetate: propionate ratio between 4 and 16-h
Acetate:propionate production ratio between 4 to 16-h (mM/mM)
No Ac
With Ac
0
1
2
3
4
5
1
2
3
4
P = 0.05
Gas Composition

CHAPTER FOUR

SUPPLEMENTATION OF SODIUM ACETATE AFFECTS IN VITRO GAS PRODUCTION AND VOLATILE FATTY ACID PROFILE

L. M. JUDD and R. A. KOHN

ABSTRACT: Understanding the regulation of rumen fermentation pathways may improve fermentation efficiency, decrease production of wasteful gases, and improve in vitro methods for studying fermentation. In theory, production rate of a specific VFA may be decreased when the concentration of the VFA increases because Gibbs energy available to drive production of the VFA is decreased by its concentration (Ungerfeld and Kohn, 2006). To test this theory, this experiment assessed the effect of sodium acetate (NaOAc, 50 mM) addition on VFA and gas profile during in vitro fermentation. Rumen fluid samples (n=16) with 1% timothy hay were incubated with or without 50 mM NaOAc addition. Tubes were equilibrated with a 50/50 gas mixture of CO_2 and N_2 and incubated at 39°C while shaking with 20-mL syringes attached to collect gases. Total volume of medium and rumen fluid was 10 mL per tube. Measurements of VFA and gas production were recorded at 0, 4, 16, 24, and 48 h. Means for treatments with acetate addition vs. control were reported when different (P <0.05). Sodium acetate addition decreased cumulative acetate production and total VFA production by 24 h ($P < 0.05$) but not production of propionate or butyrate. Sodium acetate addition decreased ratio of produced acetate to produced propionate (A:P) and ratio of produced acetate to produced butyrate (A:B). Acetate addition decreased gas production between 4 and 16 h ($P < 0.05$). NaOAc addition decreased ($P < 0.05$) acetate, propionate, and total VFA production between 0 and 4 hours, but propionate production increased ($P < 0.05$) between 24 and 48 hours. Acetate addition decreased ($P < 0.05$) ratio of produced A:P and A:B between 0 and 4 hours. The A:P ratio was close to 1 for the treatment with added acetate indicating the higher acetate concentration may have inhibited production of acetate. Gas production is

stoichiometrically linked to acetate or butyrate production, and not propionate production, and adding acetate decreased both acetate and gas production.

Key words: fermentation gases, in vitro procedures, methane, rumen fermentation, sodium acetate, volatile fatty acids

INTRODUCTION

Rumen fermentation leads to the degradation of starch, fiber, and protein. During digestion, ruminal fermentation occurs and breaks food down into short chain fatty acids that provide energy to the animal (Russell and Hespell, 1981). Dietary manipulations can cause changes to the use of energy provided by feed given the same amounts of digestible energy (Sutton, 1985) as well as to volatile fatty acid (VFA) concentrations in the rumen (Sutton et al., 2003). There is a direct correlation of acetate to propionate ratio from fermentation in the rumen with the dietary the forage: concentrate (F:C) ratio (Moss et al., 2000). VFA and gas profiles are stoichiometrically linked to methane emissions. Acetate, propionate, and butyrate are the primary VFAs produced during rumen fermentation and production of acetate, 2 CO_2, and 4 H_2 can lead to downstream synthesis of methane.

The goal of this study is to improve our understanding of the fermentation process in vitro and in vivo. In theory, production of any given VFA may be limited by accumulation of that VFA making it thermodynamically less feasible to produce more of that VFA, thereby causing a shift to different VFA. To test this theory, we hypothesize that the addition of sodium acetate to the in vitro system will shift fermentation away from acetate towards propionate or butyrate, and concomitantly decrease gas production associated with acetate production.

MATERIALS AND METHODS

Experimental procedures were approved by the University of Maryland Institutional Animal Care and Use Committee (IACUC) [850123-1].

Experimental Design and Treatments

Two treatments were compared in a completely randomized design: sodium acetate (NaOAc; 50mM added concentration) or control (no added NaOAc). Each treatment was replicated 8 times. Total volume of each treatment in the 20-mL Hungate glass tubes was 10-mL. Timothy hay (1%) was used as a substrate based on results from a previous experiment (Judd and Kohn, 2018) that did not show significant differences of substrate concentration on treatment. Rumen fluid at a concentration of 100% (9.5 mL in each tube) was used in this experiment and a 50/50 gas mixture (calculated by volume) of carbon dioxide to nitrogen (CO_2-N_2). Sodium acetate or buffered media (0.5 mL) was added to each treatment for a final volume of 10-mL.

Rumen Fluid Collection and Sample Preparation.

Timothy hay was pre-weighed (0.10 g) using a Mettler Toledo AE260 Delta Range (Columbus, OH) 4-place balance and then placed into labeled 20-mL Hungate glass tubes with a rubber stopper and screw caps. The average weight of the timothy hay was 0.1015 g (SD = 0.0005). Buffered in vitro medium was prepared, perfused with CO_2, and reduced with sodium sulfide and cysteine reducing agent (Goering and Van Soest, 1970). Rumen fluid was collected from a permanently non-lactating rumen-fistulated Holstein cow fed a timothy hay diet. Approximately 0.5 -1 L of rumen fluid (solids and liquid) was collected anaerobically in 50-mL centrifuge tubes. Rumen fluid was added to a blender that had been infused with CO_2, and blended for 20 seconds. Rumen fluid was strained through 4 layers of cheese cloth and glass wool into a 1 L flask filled with CO_2. The rumen fluid was stirred continuously with a magnetic bar and infused with 100% CO_2 while distributing to tubes and perfusing tubes with CO_2.

Each Hungate tube was sealed with a rubber stopper and screw cap, then CO_2 was removed using a Precision vacuum pump (Precision Scientific, Chicago, IL) to 0.5 atm. In each 20-mL tube, 20-mL of the 50/50 CO_2-N_2 gas mixture treatment was added using a 20-mL syringe fitted with a 27-gauge needle and 3-way stopcock (Cole-Parmer, Vernon Hills, IL). The tube was vacuumed out again, then another 20-mL of the gas mixture added. A third and final vacuum was performed, and 20-mL of the gas mixture was added, and the syringe was locked and remained in the Hungate tube. This was repeated for each gas treatment for each the in vitro tubes. The 20-mL syringes had tick marks at indicated intervals of 0.2-mL. The process to add the treatments was conducted anaerobically and in randomized order. Tubes were then given 0.5 mL of 1M acetate (NaOAc) or 0.5 mL of buffer solution (this addition increased starting concentration of acetate for the treatment by 50 mM) by inserting a 3-mL gas tight syringe with 27-gauge needle into the in vitro tube. Finally, 9.5 mL of rumen fluid was added using a 10 mL syringe attached to a 27-gauge needle to each of the 20-mL Hungate tubes. A 1 mL sample was taken for initial VFA concentration. The addition of the rumen fluid and NaOAc caused the 20-mL syringe to rise to about 10-mL, and this gas was removed from the tube before incubation in a shaker at 39 °C.

Gas Measurement

Gas production was measured, and liquid was sampled for VFA at 0, 4, 16, 24, and 48 hours. Gas production was measured in the attached syringes at 37 °C and discarded at each sampling. Gas volume was converted from mL to μmol by dividing the average gas produced for each treatment by 25,600 mL/μmol in accordance with the ideal gas law.

Liquid samples for volatile fatty acids (VFAs) were collected by inverting the in vitro tube, allowing the substrate to settle then using a 27-gauge needle and 5-mL gas tight syringe to withdraw 1 mL of sample. The sample was expelled into a 1.5 mL microcentrifuge tube and frozen in a -20°C freezer for subsequent analysis. VFA samples were prepared for GC analysis using a modified method (Erwin et al., 1961). The 1.5 mL microcentrifuge tubes containing the VFA samples were thawed at room temperature then spun in a centrifuge at 12,000 x g for 30 minutes. Supernatant (0.7 mL) was removed and phosphoric acid (0.3 mL, 10% H_3PO_4) added (De-La Rubia et al., 2009). The gas chromatograph (Hewlett-Packard model 6890) with a 4.6 m length x 0.318 cm outer diameter x 2.1 mm inner diameter packed GC column (60/80 Carboxen-1000 support, model 1-2390, Supelco, Inc, Bellefonte, PA), and flame ionization detector (FID). The split ratio of the injector port (220°C) was 100:1. Helium was used as a carrier gas with a flow of 40 mL/min. The initial column temperature was 130°C held for 10 min, then increased to 200 °C (ramp of 80°C/min) for 1 min, and a post-run temperature of 120°C. The detector temperature was 200°C with a hydrogen and air flow of 40 mL/min and 200 mL/min, respectively. VFA production is reported as the change in concentration at each interval.

Statistical Analysis

Statistical analyses were conducted using JMP Pro 11 (JMP®, Version *11*. SAS Institute Inc., Cary, NC, 1989-2007). There were two mixed models used. The first model used was: $Y_{ijk} = \mu + A_i + T_j + \varepsilon_{ijk}$ for response variables measured over time within tubes. The second was: $Y_{ij} = \mu + A_i + \varepsilon_{ij}$ for response variables measured only once. For each model, y_{ij} is the response, μ is the mean of the population, A_i is the effect of acetate, with or without 50 mM sodium acetate addition, T_j is time measured at 0, 4, 16, 24, and 48 h, and $\varepsilon_{ij(k)}$ is

the residual effect. The model was run by time which was a continuous variable. This model measured the effect of sodium acetate treatment on total gas production and VFA production over time.

RESULTS AND DISCUSSION

Effect of Sodium Acetate Addition

Sodium acetate addition (50 mM) decreased cumulative VFA production at 48 h. Sodium acetate addition also decreased ($P < 0.05$) acetate production at 0 – 4 h. (Table 4.1). The decrease in acetate production was expected based on the Second Law of Thermodynamics. For example, addition of sodium acetate would have increased product concentration, making it less thermodynamically feasible to convert glucose to acetate compared with other products (Kohn and Boston, 2000; Ungerfeld and Kohn, 2006). Cumulative propionate and butyrate production were not affected by sodium acetate addition (Table 4.1). As expected, the addition of sodium acetate caused a decrease in acetate production which led to decreased ($P < 0.05$) total VFA production (Table 4.1). This effect likely resulted from the decrease in cumulative production of acetate. Although cumulative VFA production was lower with sodium acetate addition, the profile of produced VFA was numerically different, indicating there may have been a shift away from acetate towards butyrate or propionate. This shift may be due to the acetate being thermodynamically limited (Kohn and Boston, 2000). The acetate/propionate ratio was lower ($P < 0.05$) for the sodium acetate addition treatment (Table 4.1). The decrease in the acetate/propionate ratio is a direct result of the inhibited production of acetate in the treatment.

Gas production was lower ($P < 0.05$) between 4 and 16 h with sodium acetate addition (Table 4.2). Gas profile and VFA profile are stoichiometrically linked, and the production of acetate also releases 2 CO_2 per glucose molecule converted, and leads to downstream synthesis of methane from CO_2 and H_2 (Kohn and Boston, 2000; Ungerfeld and Kohn, 2006).

The addition of sodium acetate decreased ($P < 0.01$) acetate production between 0 and 4 h (Table 4.2). There was no effect of sodium acetate addition on acetate production at any other timepoints, however this initial decrease in production may have made acetate thermodynamically limited, thus allowing for a shift in fermentation towards propionate. Addition of sodium acetate decreased ($P < 0.05$) propionate production between 0 and 4 h but increased ($P < 0.05$) propionate production between 24 and 48 h (Table 4.2). Propionate production may have been lower in the first 4 hours due to the rate of growth of propionate-producing bacteria whose optimal growth is best in lower pH and in a starch substrate (Moss et al., 1995; Russell, 1998). Timothy hay was used in this experiment as a substrate, thus selecting for the growth of acetogens. Sodium acetate addition lowered ($P < 0.01$) production of VFA between 0 and 4 h. The decrease in VFA at this time most likely is a result of the decrease of acetate production, as total VFA is a function of individual produced VFA. Production of acetate relative to propionate was lower ($P < 0.05$) with sodium acetate addition. The lower ratio of acetate production to propionate was similar to when corn is used as the substrate (Van Kessel and Russell, 1996).

Summary

This study evaluated the effect of sodium acetate addition (50 mM) on gas and VFA production in vitro. This study found that gas and VFA production are affected by the

starting concentration of sodium acetate in the system. Higher starting concentration of acetate inhibited production of acetate both cumulatively and overtime. Initial starting concentration of acetate also decreased gas production in the first 4 hours in the in vitro system. This decrease in gas production in the first four hours, along with the decrease in acetate production, suggests that there was a shift in fermentation away from acetate towards propionate or butyrate. In addition, the ratio of acetate to propionate produced was close to 1 for the treatment with added acetate, demonstrating that the production of acetate was inhibited by accumulated acetate, and suggesting that the Second Law of Thermodynamics controlled VFA profiles in this system.

To effectively develop and test a method to measure VFA and gases, future studies need to evaluate other factors that may affect the in vitro system through thermodynamic control. These factors include other volatile fatty acids or specific gases that can shift fermentation, as well as factors that can affect the available pathways for substrate synthesis such as presence of certain bacterial populations or substrates.

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The authors thank Rachel Rha and Jillian Yant for their assistance in conducting this study.

Table 4.1. Main effect of sodium acetate on gas and VFA production

	Treatment			
	Acetate (mM)[1]			
Gas Production	**No**	**Yes**	**SEM**	***P***
Total Gas (μmol)[2]	434	393	49.2	0.56
Final Gas (μmol)[3]	18	4	6.3	0.12
Total VFA Production				
Acetate (mM)	45[a]	27[b]	5.2	< 0.05
Propionate (mM)	16	17	0.7	0.33
Butyrate (mM)	6.9	7.4	0.21	0.13
Total VFA (mM)	72[a]	56[b]	5.4	< 0.05
Acetate/Propionate (mM/mM)[4]	2.8[a]	1.6[b]	0.29	< 0.05
Acetate/Butyrate (mM/mM)	6.5[a]	3.7[b]	0.67	< 0.05

[a,b]Within a row, means without a common superscript differ (P < 0.05)

[1] Acetate treatment indicates the addition of sodium acetate (NaOAc); N: no acetate, Y: 50 mM NaOAc

[2] Gas is the production of gas between time points; 4 (between 0 and 4 hours), 16 (between 4 and 16 hours), 24 (between 16 and 24 hours), 48 (between 24 and 48 hours)

[3] Final gas is the total gas produced from time 0 to the given time point

[4] Initial Rumen Fluid Acetate: Propionate Ratio = 3.26

Table 4.2. Effect of acetate on the production of gas and VFAs by time[1]

	Treatment			
	Acetate (mM)[2]			
	N	**Y**	**SEM**	***P***
Gas (μmol)				
Initial Concentration (T = 0)	0	0	--	--
0-4 h	261	266	42.3	0.94
4-16 h	134[a]	104[b]	9.2	< 0.05
16-24 h	21	20	6.8	0.92
24-48 h	18	4	6.3	0.12
Acetate (mM)				
Initial Concentration (T = 0)	106[b]	190[a]	2.9	< 0.01
0-4 h	17[a]	-5[b]	4.6	< 0.01
4-16 h	15	13	4.4	0.73
16-24 h	6	10	3.3	0.34
24-48 h	7	9	2.2	0.56
Propionate (mM)				
Initial Concentration (T = 0)	34[a]	32[b]	0.3	< 0.01
0-4 h	6[a]	4[b]	0.5	< 0.05
4-16 h	5.5	5.6	0.78	0.95
16-24 h	2.2	3.6	0.59	0.12
24-48 h	3.0[a]	4.4[b]	0.48	< 0.05
Butyrate (mM)				
Initial Concentration (T = 0)	15	15	0.1	0.81
0-4 h	2.5	2.1	0.19	0.22
4-16 h	2.7	2.5	0.34	0.77
16-24 h	0.8	1.3	0.29	0.24
24-48 h	1.0	1.4	0.25	0.22
Total VFA (mM)				
Initial Concentration (T = 0)	162[b]	243[a]	3.2	< 0.01
0-4 h	27[a]	2[b]	4.8	< 0.01
4-16 h	25	22	5.6	0.76
16-24 h	9	16	4.2	0.29
24-48 h	12	16	3.0	0.38
Acetate/Propionate (mM/mM)[3]				
0-4 h	3[a]	-1[b]	1.2	< 0.05

4-16 h	2.8	5.2	1.62	0.31
16-24 h	2.4	1.8	0.65	0.52
24-48 h	2.1	1.5	0.53	0.44
Acetate/Butyrate (mM/mM)				
0-4 h	7[a]	-4[b]	3.2	< 0.05
4-16 h	6	11	3.7	0.28
16-24 h	1.8	-1.3	6.38	0.74
24-48 h	6.5	-3.7	7.24	0.34

[a,b]Within a row, means without a common superscript differ ($P < 0.05$)
[1]VFA production is calculated as the change is concentration between each interval
[2]Acetate treatment indicates the addition of sodium acetate (NaOAc); N: no acetate, Y: 50 mM NaOAc. Initial Rumen Fluid Acetate: Propionate Ratio = 3.26

CHAPTER FIVE

EFFECT OF PROBIOTICS ON PRODUCTION OF RUMEN FERMENTATION IN DAIRY COWS: A REVIEW

L. M. JUDD and R. A. KOHN

INTRODUCTION

Direct fed microbials (DFM) have become a primary focus in livestock as antibiotic resistance increases as they may have the ability to alter fermentation activity and shift fermentation to other byproducts in the rumen (McAllister et al., 2011). Lactic acid producing bacteria are believed to stimulate the growth of bacteria that use lactic acid (McAllister et al., 2011) and *Propionibacterium* are natural producers of propionate (Vyas et al., 2014). Propionate producing and lactate producing bacteria may serve as a competitor whether direct or indirect with bacteria that primarily lead to acetate production. The use of lactic acid producing bacteria have a very important role in the potential to shift rumen fermentation. Lactic acid producing bacteria can be converted to propionate. *Lactobacillus* and *Bifidobacterium* are the major genera that probiotics are made from (Sorokulova, 2013). There have been several studies that have addressed the efficacy of different bacterial species in ruminal fermentation (McAllister et al., 2011). Individual studies have investigated the efficacy of direct fed microbials in vitro and have seen promising effects of the various treatments. However, when the same treatment is given in vivo the treatment effect is generally not observed. Therefore, the purpose of this review is to evaluate both in vivo and in vitro studies that have utilized lactic acid bacteria or direct fed microbials as a probiotic supplement to potentially identify potential mechanisms that can enhance our understanding of how each of these supplements work as well as to determine potential guidelines or dosage limitations for the use of these supplements.

METHODS

The literature search was conducted using three primary journal databases: The

Journal of Dairy Science, The Journal of Animal Science, and The Journal of Animal Feed and Technology. The search ranged from papers published between 1985 – 2016. The following terms were entered in the search engine of each journal website: lactic acid bacteria (LAB), *Lactobacillus*, *Enterococcus*, direct fed microbials (DFM), and rumen (this term was only used in the Journal of Animal Science and the Journal of Animal Feed and Technology). There were 38 papers identified that fit our search criteria. Table 1 depicts the overall distribution of the studies based on experiment type. Table 2 illustrates the distribution of the studies based on the response measured in the experiment. These studies were then broken down by method type: in vivo (Table 3), in vitro (Table 4), and studies using both methods (Table 5). Based on these response variables the papers were further categorized into two main categories to be discussed further: 1) production, digestibility, and intake and 2) volatile fatty acids and gases. The 38 papers and treatment combinations used in the studies are listed in table 6.

DISCUSSION

Production, Digestibility, and Intake

Body weight gain in calves has been one primary observation when evaluating the efficacy of lactic acid bacteria supplementation. Average daily gain is one production parameter measured in several studies. One study (Cruywagen et al., 1996) used 1 ml (5×10^7) of *Lactobacillus acidophilus* as a supplement in calves along with milk replacer. The bacteria were supplemented in the milk replacer. These calves increased body weight gain during the first two weeks in comparison with calves receiving no supplement. These findings are similar with other studies (Vasconcelos et al., 2007; Frizzo et al., 2010; Kenney et al., 2015) that also found live weight or average daily gain increases in cattle. In one study, (Frizzo et al., 2010) calves were given a diet that included a LAB mixture

consisting of two lactobacillus strains (*L. casei* and *L. salivarius*; 10^9 CFU/kg live weight) as well as *Pediococcus acidilactici* (10^9 CFU/kg live weight). The treatment was fed orally to calves. The only observed affect in the study was the growth performance of the calves. *Lactobacillus acidophilus* was utilized in the Vasconcelos et al., 2007 and Kenney et al., 2015 studies. Both studies also utilized a *Propionibacterium* as part of their supplement. Vasconcelos et al., 2007 observed that although *L. acidophilus* combined with *P. freudenreichii* tended to increase the live body weight gain (with decreasing *L. acidophilus*) in the steers there was no treatment effect on the average daily gain (ADG) of these animals. The LAB was introduced as a mix in with the water in this study. There were three different concentrations (CFU/(steer·day)) of *L. acidophilus* used in this study: Low (1×10^7), Medium (M; 1×10^8), and High (H; 1×10^9). The concentration of *P. freudenreichii* was consistent in each treatment at 1×10^9 CFU/(steer·day). The study by Kenney et al., 2015 included an *Enterococcus faecium* species in addition to the *Propionibacterium* and *L. acidophilus*. In this study, researchers noted that in the first experiment there was an increase in the initial dry matter intake (DMI) which could have potentially led to observed ADG effect. The treatments in this study consisted of a lactate-producing (*Enterococcus faecium* and *Propionibacterium*; 10^9 CFU/d) combination and a lactate-utilizing combination (*Propionibacterium* and *L. acidophilus*; 10^9 CFU/d). The cows were also fed a corn silage and haylage mix in their diet. These DFM's were mixed into ground corn given as a top-dress to the steers. Another study, (Basso et al., 2014) observed in lambs an increase in average daily gain. These lams were fed a corn silage and the bacteria were applied to the silage in this study. Two *Lactobacillus* strains (*L. buchneri*; 1×10^5) and (*L. plantarum*; 1×10^5) were sprayed onto fresh forage with a constant mixing. There was an

increase in DMI in this study as well which is like other studies. None of these previously mentioned studies used any yeast supplementation in their experiments.

There were three studies (Nocek et al., 2003; Nocek and Kautz, 2006; Oetzel et al., 2007) that observed effects of DFM on milk production factors. The Nocek studies used *S. cerevisiae* (commercially made Biomate yeast plus) (Nocek et al., 2003) in addition to the *E. faecium* microbial species. Nocek et al., 2003 evaluated the effect of *E. faecium* and yeast during the pre- and post-partum periods. The Holsteins were given 90 g/d of the supplement which contained 5×10^9 CFU of both yeast and bacteria. The researchers observed that the supplementation of the Biomate and *E. faecium* an increase in DMI, milk yield, and milk protein in the postpartum cows. Similar to these findings, one study (Nocek and Kautz, 2006) also observed an increase in DMI and milk, however they also saw a decrease in milk fat percentage. This study utilized a concentration of 5×10^9 CFU of the Biomate and *E. faecium*. The amount of the supplement differed in this study as they only gave the Holsteins 2 g/d. Both studies had diets that contained corn silage and mixed haylage. The third study (Oetzel et al., 2007), had a few different observations in regard to milk production. This study used a DFM that had *E. faecium* and *S. cerevisiae* with the same concentration (5×10^9 CFU) as the previously mentioned studies. The amount given to each cow was 2 g/d during the pre- and post-partum periods. There was no silage provided in this study. There was an increase in milk fat percentage in the first lactation cows. This finding differs from a previous study (Nocek and Kautz, 2006). Interestingly, there was an increase in milk protein percentage in the second and greater lactation cows, which is similar to findings in Nocek et al., 2003. Two other studies, one using feedlot cattle and barley silage (Beauchemin et al., 2003) and one in vivo using growing bulls with

no silage (AlZahal et al., 2014) both used *S. cerevisiae* in addition to *E. faecium* but there was no effect of probiotic treatment in either of these studies.

Heifer growth and silage preservation were evaluated in a study (Cleale IV et al., 1990) using *Pediococcus acidilactici* and *L. xylosus*. Holsteins were fed corn silage that was inoculated with a commercial product, AgMaster™ containing *Pediococcus acidilactici* and *L. xylosus* containing 2×10^5 CFU/g of forage. There was an increase in DMI for the heifers given the supplement and BW gain increased during the last half of the experiment (84 days total) although feed efficiency was lower in these heifers. The OM (organic matter) digestibility, ADF, and N were higher in the heifers fed the silage that was inoculated. An in vitro study was conducted to evaluate the difference in digestibility between wheat and corn silages (Weinberg et al., 2007). This study used rumen fluid, ten different bacterial treatments (10^6 CFU/g) from commercial products, and starch (for concentrate based feed). The bacteria used in this study were: two species of *L. plantarum* (Ecosyl, Yorkshire, UK and Agri-King, Fulton, IL), two species of *Pedioccoccus pentosaceus* (Ecosyl, Yorkshire, UK and Agri-King, Fulton, IL), a *L. pentosus* (Agri-King, Fulton, IL), two species of *E. faecium* (both from Agri-King, Fulton, IL), two species of *L. buchneri* (Biotal, Milwaukee, WI; Pioneer, Des Moines, IA), and a combination of *L. plantarum* and *E. faecium* (Pioneer, Des Moines, IA). The starch was given at a ratio of 1:2 (starch:silage) or 2:1 (starch: silage). The researchers observed that treatments that had pre-inoculated wheat or corn silages increased dry matter digestibility (DM-D) and NDF digestibility. In the starch treatments with the pre-inoculated corn or wheat silages NDF digestibility was decreased. There was a study that had similar findings to the effect of LAB on silages as the previous paper (Kenney et al., 2015). Organic matter digestibility

was increased in one study (Ellis et al., 2016). This study was conducted in vitro and used Lactobacillus species (*L. lactis, L. plantarum, L. buchneri*) as well as *E. faecium* at concentrations ranging from 10^6 CFU/g – 5×10^6 CFU/g. Silages used in this study were rye grass, grass/clover, and maize. The treatments containing all of the LAB were most effective in the grass silages. Several other in vitro studies observed increases in dry matter digestibility (DMD) (Jalč et al., 2009a; Cao et al., 2011; Sanchez et al., 2014; Babaeinasab et al., 2015). Sanchez et al., 2014 utilized *Propionibacterium acidipropionici* as the probiotic at a concentration of 6×10^6 CFU/g in addition to extrusa grass and sorghum hay as silages. This experiment was conducted both in vitro and in vivo (feedlot cattle). Combinations of *L. plantarum*, *L. buchneri*, and *P. acidipropionici* were used in one study (Babaeinasab et al., 2015) in addition to a potato wheat straw silage. The treatments containing the silage had higher DMD and concentration of probiotic was 3×10^6 CFU/g. Similar to the previous study, *L. plantarum* was used by researchers (Cao et al., 2011) at a concentration of 10^5 CFU/g with cabbage and lettuce as the silages. Jalc et al., 2009 was the only study in this group to also use *E. faecium* (10^9 CFU/mL) as a probiotic. Both DMD and OM degradability were increased with the treatment using grass silage. In a different study using *L. plantarum*, *L. fermentum,* and *E. faecium* and corn silage (Jalč et al., 2009b), the researchers observed an increase in OM degradability as well as an increase in NDF (neutral detergent fiber) when using the LAB with a concentration of 10^9 CFU/mL. Several studies that used LAB without silage had no treatment effects of the probiotic in bulls (Abu-Tarboush et al., 1996), calves (Bayatkouhsar et al., 2013), growing cattle (Higginbotham and Bath, 1993), and growing goats (Whitley et al., 2009). Interestingly, several studies that used silage and LAB had no treatment effects. This occurred in both feedlot cattle,

when using corn (Elam et al., 2003) or barley(Ghorbani et al., 2002), as well as growing cattle with barley (Vyas et al., 2014), and in lactating cattle using corn silage (Raeth-Knight et al., 2007) or maize (Meeske and Basson, 1998).

Volatile Fatty Acids and Gases

Volatile fatty acid profile, gas production, and pH can provide insight into rumen fermentation and methane production. The purpose of using LAB generally is to increase production of propionate which may reduce methane production as both propionate and methane hydrogen sinks. Compared to in vivo studies, in vitro studies using probiotics quite often have treatment effects even at low concentration of probiotic. There were two studies that used *P. acidipropionici* (Sanchez et al., 2014; Babaeinasab et al., 2015) that had effects in vitro. The Sanchez et al., 2014 study used 6×10^6 CFU/g of probiotic and found that supplement of probiotic increased total VFA production, as well as increasing propionate, decreasing acetate production and subsequently decreasing the acetate/propionate ratio. Babaeinasab et al., 2015 observed that a combination of *P. acidipropionici, L. buchneri, and L. plantarum* (3×10^6 CFU/g) increased pH, lactic acid, and propionate, and decreased acetate/propionate ratio. The increase in pH is similar to what was observed in two in vivo studies on lactating dairy cattle. One study used corn or haycrop silage with *E. faecium, L. plantarum,* and *S. cerevisiae* (10^5CFU/mL) (Nocek et al., 2002) whereas the other used *E. faecium, Lactococcus lactis,* and *S. cerevisiae* (10^9CFU/g) with corn or grass silage (Chiquette et al., 2015). Other in vitro studies however, observed decreases in pH when LAB was used. One study (Weinberg et al., 2004), which used combinations of *L. plantarum, Pediococcus pentosaceus, L. pentosus, E. faecium,* and *L. buchneri* (10^6CFU/g). Another study (Amado et al., 2012), used four

different concentrations of probiotic (10^6 CFU/g, 10^8 CFU/g, 3×10^5 CFU/g, and 5×10^5 CFU/g) mixtures containing *L. plantarum, Lactococcus lactis, Pediococcus acidilactici, L. buchneri,* and *E. faecium.* Lactic acid was increased in this study, and in addition to the decrease in pH there was also a decrease in acetate. Lactic acid was increased in one study (Cao et al., 2011) using *L. plantarum* (10^5 CFU/g) with a decrease in both pH and methane. *L. plantarum* and *L. brevis* (10^6 CFU/g) were used in one study (Parvin et al., 2010) and increased both lactic acid and acetic acid. Acetic and propionic acid were increased in herbage that was treated with 10^6 CFU/g of combinations of *L. plantarum, E. faecium, Pediococcus acidilactici, L. salivarius,* and *L. buchneri* in one study (Keles and Demirci, 2011). This silage was fed to lambs and found to increase intake and decreased lactic acid. Propionate production was increased in one study using grass silage (Jalč et al., 2009a), and acetate and butyrate were both decreased, however there was also a decrease in total VFA produced. One study had opposite effects of two different probiotics on methane (Jeyanathan et al., 2016). This study was conducted both in vitro and in vivo used three different probiotics: *L. bulgaricus, L. pentosus,* and *P. freudenreichii*. Both the *L. bulgaricus* and *L. pentosus* used a concentration of 3×10^{10} CFU/animal in vivo and the *P. freudenreichii* was supplemented at 6×10^{10} CFU/animal. The *L. pentosus* decreased methane production and the *P. freudenreichii* increased methane production. There were two in vitro studies that did not have an effect on VFA production however one study increased microbial biomass yield (Contreras-Govea et al., 2011), when using *L. plantarum, E. faecium, L. pentosus,* and *Lactococcus lactis* as supplements at a concentration of 10^6 CFU/g. Each of the LAB were used as individual treatments and were not combined. The other study (Dawson et al., 1990), used a mixture of *L. acidophilus*

(1.2 – 2.3 × 10^9 CFU/g), *E. faecium* (1.5 – 2.6 × 10^{10} CFU/g) in combination with live *S. cerevisiae* (1.4 – 2.7 × 10^9 CFU/g) or *S. cerevisiae* alone (1.4 – 4.2 × 10^9 CFU/g). The study also used an inactive, killed yeast as a separate treatment. This study found that the live yeast treatments increased cellulolytic organisms in vitro. Tables 6 – 9 provide summaries for the studies and treatment effect for all studies (Table 6), in vivo (Table 7), in vitro (Table 8), and in studies that were conducted both in vitro and in vivo (Table 9).

Summary

These studies found using the literature search demonstrate that there is a greater need for increased research using probiotics, especially in vivo studies. Although most in vitro studies have effects on rumen fermentation parameters (VFA and gas production), this effect is not often observed in vivo. Table 10 depicts the significance of these studies based on specific responses measured (production or rumen fermentation parameters) and demonstrates that of the 38 studies found that none of the paraments are significant in greater than 50% of studies. Although milk production was found to be significant 50 % of the time, there were only 6 studies that evaluated milk production. *E. faecium* and *Lactobacillus* species generally had treatment effects both in vitro and in vivo and should be studied further in future studies to elucidate the effect on rumen fermentation.

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Table 5.1. Distribution of study type using LAB

Technique	Number of Studies	Number of Experiments	Total Number of Treatments
In vitro	12	19	195
In vivo	20	27	72
Both	6	12	41
Total	38	58	308

Table 5.2. Distribution of studies utilizing LAB

Measurement	Number of Studies	Number of Experiments	Total Number of Treatments
Production	8	8	22
Digestibility	13	16	67
Intake	24	32	88
ADG	14	20	55
VFA Concentration	21	32	210
VFA Production	5	10	87
CH_4 Production	6	8	75
CH_4/Total gas	2	3	31

Table 5.3. Distribution of in vivo studies utilizing LAB by response

	In vivo		
Measurement	**Number of Studies**	**Number of Experiments**	**Number of Treatments**
Production	7	7	19
Digestibility	6	7	19
Intake	19	26	71
ADG	9	15	41
VFA Concentration	6	7	22
VFA Production	1	1	4
CH_4 Production	1	1	4
CH_4/Total gas	-	-	-

Table 5.4. Distribution of in vitro studies utilizing LAB by response

	In vitro		
Measurement	**Number of Studies**	**Number of Experiments**	**Number of Treatments**
Production	1	1	3
Digestibility	4	5	36
Intake	2	2	6
ADG	2	2	6
VFA Concentration	10	17	162
VFA Production	4	9	83
CH_4 Production	5	7	71
CH_4/Total gas	1	2	25

Table 5.5. Distribution of studies utilizing LAB with both in vivo and in vivo techniques by response

Measurement	Number of Studies	Number of Experiments	Number of Treatments
Production	-	-	-
Digestibility	3	4	12
Intake	3	4	11
ADG	3	3	8
VFA Concentration	5	8	26
VFA Production	-	-	-
CH_4 Production	-	-	-
CH_4/Total gas	1	1	6

Table 5.6. Lactobacillus, yeast, and silage treatments by study

Author	LAB[1]	Yeast	Silage (Yes/No)
Abu-Tarboush et al., 1996	*L. acidophilus, L. plantarum*	No	No
AlZahal et al., 2014	*E. faecium*	*S. cerevisiae*	Yes
Amado et al., 2012	*L. plantarum, Lactococcus lactis, Pediococcus acidilactici, L. buchneri, E. faecium*	No	Yes
Arriola et al., 2011	*Pediococcus pentosaceus, P. freudenreichii, L. buchneri*	No	Yes
Babaeinasab et al., 2015	*L. buchneri, L. plantarum, P. acidipropionici*	No	Yes
Basso et al., 2014	*L. buchneri, L. plantarum*	No	Yes
Bayatkouhsar et al., 2013	*L. acidophilus, L. rhamnosus, L. casei, L. delbrueckii, Bifidobacterium bifidum, E. faecium*	No	No
Beauchemin et al., 2003	*E. faecium*	*S. cerevisiae*	Yes
Cao et al., 2011	*L. plantarum,*	No	Yes
Chiquette et al., 2015	*E. faecium, Lactococcus lactis*	*S. cerevisiae*	Yes
Cleale et al., 1990	*Pediococcus acidilactici, L. xylosus*	No	Yes
Contreras-Govea et al., 2011	*L. plantarum, E. faecium, L. pentosus, Lactococcus lactis*	No	Yes
Cruywagen et al., 1996	*L. acidophilus*	No	No
Dawson et al., 1990	*L. acidophilus, E. faecium*	*S. cerevisiae*	No
Elam et al., 2003	*L. acidophilus, P. freudenreichii*	No	Yes/ No
Ellis et al., 2016	*Lactococcus. lactis, L. plantarum, L. salivarius, E. faecium, L. buchneri*	No	Yes
Frizzo et al., 2010	*L. casei, L. salivarius, Pediococcus acidilactici*	No	No
Ghorbani et al., 2002	*Propionibacterium, E. faecium*	No	Yes
Higginbotham et al., 1993	*L. acidophilus, Streptococcus faecium*	No	No
Jalč et al., 2009	*E. faecium, L. fermentum, L. plantarum*	No	Yes
Jalč et al., 2009	*L. plantarum, L. fermentum, E. faecium*	No	Yes
Jeyanathan et al., 2016	*L. bulgaricus, L. pentosus, P. freudenreichii*	No	No

Keles et al., 2011	*L. plantarum, E. faecium, Pediococcus acidilactici, L. salivarius, L. buchneri*	No	Yes
Kenney et al., 2015	*L. acidophilus, E. faecium, Pediococcus acidilactici, L. brevis, L. plantarum*	No	Yes
*Kristensen et al., 2010	*L. pentosus, L. buchneri, Pediococcus pentosaceus*	No	Yes
Meeske et al., 1998	*L. plantarum, L. bulgaricus, L. acidophilus*	No	Yes
Nocek et al., 2002	*E. faecium, L. plantarum*	*S. cerevisiae*	Yes
Nocek et al., 2003	*E. faecium*	*S. cerevisiae*	Yes
Nocek and Kautz, 2006	*E. faecium*	Biomate	Yes
Oetzel et al., 2007	*E. faecium*	*S. cerevisiae*	No
Parvin et al., 2010	*L. plantarum, L. brevis*	No	Yes
Raeth-Knight et al., 2007	*E. faecium, L. acidophilus, P. freudenreichii*	No	Yes
Sanchez et al., 2014	*P. acidipropionici*	No	Yes/ No
Vasconcelos et al., 2007	*L. acidophilus, P. freudenreichii*	No	Yes
Vyas et al., 2014	*P. acidipropionici, P. jensenii*	No	Yes
Weinberg et al., 2004	*L. plantarum, Pediococcus pentosaceus, L. pentosus, E. faecium, L. buchneri*	No	Yes
Weinberg et al., 2007	*L. plantarum, Pediococcus pentosaceus, L. pentosus, E. faecium, L. buchneri*	No	Yes
Whitley et al., 2014	*L. acidophilus, E. faecium, B. subtilis, Aspergillus oryzae*	No	No

*Excluded

Table 5.7. Effect of LAB supplementation in in vivo studies

Author	Summary of effect
Abu-Tarboush et al., 1996	No treatment effect
AlZahal et al., 2014	No treatment effect
Arriola et al., 2011	No treatment effect
Bayatkouhsar et al., 2013	No treatment effect
Beauchemin et al., 2003	No treatment effect
Chiquette et al., 2015	EFSC ↑ pH in SARA; prevented milk ↓ in SARA
Cleale et al., 1990	↑ OM digestibility, DMI, and BW gain; ↓ feed efficiency
Cruywagen et al., 1996	↑ ADG in 2nd week for calves
Elam et al., 2003	No treatment effect
Frizzo et al., 2010	↑ LW, LWG, and starter intake
Ghorbani et al., 2002	No treatment effect
Higginbotham et al., 1993	No treatment effect
Nocek et al., 2002	↑ pH until threshold was reached
Nocek et al., 2003	↑ DMI, milk yield, milk protein in postpartum cows
Nocek and Kautz, 2006	↑ DMI and milk; ↓milk fat %
Oetzel et al., 2007	↑ milk fat % in 1st lactation cows; ↑ milk protein % in 2 or more lactation cows
Raeth-Knight et al., 2007	No treatment effect
Vasconcelos et al., 2007	↑ live BW gain
Vyas et al., 2014	No treatment effect
Whitley et al., 2014	No treatment effect

Table 5.8. Effect of LAB supplementation on in vitro studies

Author	Summary of effect
Amado et al., 2012	↑ lactic acid and residual sugar; ↓ pH and acetic acid
Babaeinasab et al., 2015	PWSS ↑ DM, ADL, WSC, pH, ammonia-N; PWSS ↓ CP, ash free NDF, lactic acid, VFAs; PWSS + molasses and LAB ↑ CP, lactic and acetic acid, propionic acid, ↓ A:P ratio
Cao et al., 2011	↑ DM digestibility and lactic acid; ↓pH and methane
Contreras-Govea et al., 2011	↑ microbial biomass yield
Ellis et al., 2016	↑ OM digestibility (more effective in grass silage)
Jalč et al., 2009	↑ DM and OM degradability, propionate production, CLA ↓ biohydrogenation (GSLP diet); ↓ total VFA, acetate and butyrate, biohydrogenation (GSEF and GSLF diets)
Jalč et al., 2009	↑ NDF and OM degradability, total VFA production, acetate and butyrate production; ↓ ammonia N
Keles et al., 2011	↑ silage acetic and propionic acid; ↓ lactic acid and water-soluble carbohydrates; ↑ silage and total intake in lambs
*Kristensen et al., 2010	No treatment effect
Parvin et al., 2010	↑ lactic and acetic acid
Weinberg et al., 2004	↓ pH incubated sample; heat sterilized RF had lactic acid
Weinberg et al., 2007	potential to ↑ DM-D and NDF-D

Table 5.9. Effect of LAB supplementation on studies using both in vitro and in vivo methods

Author	Summary of effect
Basso et al., 2014	↑ ADG, lactic acid concentration, ↓ A:P (LBLP); ↑ pH (LB); ↑ DMI, OM, CP, NDF and carbohydrates, ↓ digestibility of OM, DM (LBLP & LB)
Dawson et al., 1990	live yeast ↑ cellulolytic organisms
Jeyanathan et al., 2016	↓ methane production (*L. pentosus);* ↑ methane production (*Propionibacterium)*
Kenney et al., 2015	↑ initial DMI, ADG (exp1); tendency to ↑ molar proportion of propionate and tendency to ↓ molar acetate (exp2)
Meeske et al., 1998	No treatment effect
Sanchez et al., 2014	↑ in vitro DMD and total VFA (PA); ↓ acetate and A:P ↑ propionate (P169) (exp 1)

Table 5.10. Treatment responses measured versus significant effect of treatment

	Measured (No. of Studies)	Significant (No. of Studies)	% Significant
Response Measured			
DMI	22	4	18.2
Gain (ADG, LW, BW)	14	5	35.7
Milk Production	6	3	50.0
Acetate	19	8	42.1
Propionate	19	4	21.1
A:P	17	3	17.6
Methane	6	2	33.3

CHAPTER SIX

THE EFFECT OF LACTIC ACID BACTERIA AND SODIUM ACETATE ON IN VITRO FERMENTATION

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ABSTRACT: The production of methane in ruminants is a greenhouse gas concern. Rumen fermentation produces three main volatile fatty acids (VFAs): acetate, propionate, and butyrate. Rumen fermentation leads to downstream synthesis of methane from the production of 2 acetate, 2 CO_2 and 4 H_2 molecules per molecule of glucose. Lactic acid bacteria (LAB) and direct fed microbials (DFM) have been used in vivo and to increase feed efficiency and production in dairy cows and have been shown to be highly effective in vitro. This experiment assessed the effect of LAB supplementation in the presence and absence of sodium acetate (NaOAc, 50 mM) on VFA and gas profile during in vitro fermentation. Rumen fluid samples (n = 24) with probiotic (10^6 CFU/mL) and 2% substrate (1% timothy hay and 1 % ground corn) were incubated with or without 50 mM NaOAc addition. Tubes were equilibrated with a 50/50 gas mixture of CO_2 and N_2 and incubated at 39°C while shaking with 20-mL syringes attached to collect gases. Total volume of medium and rumen fluid was 10 mL per tube. Probiotic treatments were:1) control for *Enterococcus faecium* (Control EF), 2) control for *Lactobacillus pentosus* (LAB)*, Selenemonas ruminantium* (lactic acid utilizer) mix (Control LS), 3) *Enterococcus faecium* (EF), and 4) *Lactobacillus pentosus, Selenemonas ruminantium* mix (LS). Each treatment was replicated in 4 tubes with repeated measures of VFA and gas volume taken at 0, 4, 16, 24, and 48 hours. Means for treatments were reported when different ($P < 0.05$). *Enterococcus faecium* increased ($P < 0.05$) gas production between 4 and 16 h. *Lactobacillus pentosus* and *Selenomonas ruminantium* increased ($P < 0.05$) production of butyrate between 0 to 4 h and tended to decrease ($P < 0.10$) the ratio of produced acetate: butyrate (A: B) between 0 to 4 h, and significantly decreased ($P < 0.05$) A: B between 24 and 48 h. *Lactobacillus pentosus* and *Selenomonas ruminantium* tended to increase ($P <$

0.10) propionate production between 0 and 4 h. Sodium acetate addition increased ($P < 0.05$) gas production between 4 to 16 h, but decreased ($P < 0.05$) gas production between 24-48 h. Sodium acetate addition tended to increase ($P < 0.10$) cumulative propionate (0 to 48 h) and propionate between 4 to 16 h. Sodium acetate addition tended to decrease ($P < 0.10$) the ratio of produced A: B between 4 to 16 h. The higher starting acetate concentration may have inhibited the production of acetate. The production of gas is stoichiometrically linked to VFA production. The addition of *Lactobacillus pentosus* and *Selenomonas ruminantium* decreased gas production and tended to increase propionate production and butyrate. *Enterococcus faecium* increased gas production but tended to decrease butyrate production.

Key words: fermentation gases, in vitro procedures, lactic acid bacteria, rumen fermentation, sodium acetate, volatile fatty acids

INTRODUCTION

Rumen fermentation results in the production of three main volatile fatty acids (VFAs): acetate, propionate, and butyrate, via the uptake of glucose. The pathway that produces 2 acetate, 2 CO_2 and 4 H_2 molecules per molecule of glucose is the most thermodynamically favorable in the rumen, and therefore most glucose is utilized this way (Ungerfeld and Kohn, 2006). Lactic acid bacteria (LAB) stimulate the bacteria that utilize lactic acid (McAllister et al., 2011). LAB and other direct fed microbials (DFM) have been shown in vivo to increase milk production parameters (milk yield, milk fat, milk protein) of dairy cows (Nocek et al., 2003; Nocek and Kautz, 2006; Oetzel et al., 2007). In vitro studies using LAB and probiotics have also been shown to have antimethanogenic properties (Cao et al., 2011). The use of LAB or DFM as supplements in dairy cows may lead to a shift in fermentation away from acetate towards more favorable end products (propionate or butyrate), thus leading to a reduction in methane synthesis.

The goal of this study is to enhance our understanding of the effect of lactic acid bacteria on fermentation in vitro and in vivo. We hypothesize that the addition of LAB and NaOAc will inhibit the pathway for acetate production by increasing the uptake of H_2 and shifting fermentation towards propionate production. To test our hypothesis, we evaluate the effect of LAB supplementation in the presence and absence of sodium acetate (NaOAc) on VFA and gas profile during in vitro fermentation.

MATERIALS AND METHODS

Experimental procedures were approved by the University of Maryland Institutional Animal Care and Use Committee (IACUC) [850123-1].

Experimental Design and Treatments

The experimental design was a 4 × 2 factorial CRD; the treatments were probiotic supplementation (control for *Enterococcus faecium* (Control EF), control for *Lactobacillus pentosus* (LAB)*, Selenemonas ruminantium* (lactic acid utilizer) mix (Control LS), *Enterococcus faecium* (EF), and *Lactobacillus pentosus, Selenemonas ruminantium* mix (LS); 10^6 CFU/mL) and sodium acetate (50mmol NaOAc addition or not). Total volume of each treatment was 10 ml per 20-ml Hungate tube. Each treatment was replicated 4 times. The concentration of substrate used was 2% (1% timothy hay (0.10 g) and 1 % ground corn (0.10 g). Rumen fluid at a concentration of 100% was used in this experiment to focus on the effect of probiotic and acetate.

Rumen Fluid Collection and Sample Preparation

Timothy hay and ground corn were pre-weighed (0.10 g each) into labeled 20-mL Hungate glass tubes with a rubber stopper and screw caps. The Timothy hay and ground corn were measured on a Mettler Toledo AE260 Delta Range (Columbus, OH) 4-place balance. The average weight for the 0.10 g Timothy hay, ground corn, and total substrate was 0.104 g (SD = 0.003), 0.103 g (SD = 0.002), and 0.207 g (SD = 0.003), respectively. The in vitro medium buffered medium was prepared, perfused with CO_2, and reduced with a reducing agent (sodium sulfide and cysteine) as previously published (Goering and Van Soest, 1970). Rumen fluid was collected according to the IACUC protocol from a permanently non-lactating rumen-cannulated cow consuming a timothy hay diet. Approximately 0.5 -1 L of rumen fluid (solids and liquid) was collected anaerobically in 50-mL centrifuge tubes. Rumen fluid was infused with CO_2 and blended for 20 seconds.

Rumen fluid was strained through 4 layers of cheesecloth and glass wool into a 1-L flask infused with CO_2. The strained rumen fluid was infused with CO_2 and stirred continuously with a magnetic bar.

The Hungate tubes were sealed with rubber stoppers and screw caps. Air was removed from each tube using a Precision vacuum pump (Precision Scientific, Chicago, IL) to 0.5 atm. In each 20-mL tube, 20 ml of 50/50 CO_2-N_2 gas mixture was added in random order using a 20-mL gas-tight syringe fitted with a 27-gauge needle and 3-way stopcock (Cole-Parmer, Vernon Hills, IL). The tube was vacuumed, then another 20 ml of the gas mixture added. A third and final vacuum was performed, and 20 ml of the gas treatment was added, and the syringe locked with a 3-way stopcock and remained in the Hungate tube. The 20-mL syringes had tick marks indicated intervals of 0.2-mL. The process to add the treatments was conducted anaerobically and 9 mL of rumen fluid was added to each tube in randomized order. Due to the increase in pressure above 1 atm, the 10-mL syringe was not immediately removed during the addition of rumen fluid. The addition of the rumen fluid and NaOAc caused the 20-mL syringe to rise to 10 ml, the 20 mL syringe was then pressed down, so the excess air was expelled back into the 10-mL syringe. The 10-mL syringe and needle were then removed, and the air was expelled. Tubes were then given either 0.5 mL of 1M acetate (NaOAc) (this increased starting concentration of acetate for these treatments by 50 mM), or 0.5 mL of in vitro medium by inserting a 3-mL gas-tight syringe with 27-gauge needle into the in vitro tubes containing rumen fluid (9 mL) was added in random order using a 10 mL gas-tight syringe attached to a 27-gauge needle to each 20-mL Hungate tubes. Probiotic treatments were added in a Bactron IV anaerobic chamber (Labgard Class II, Type A/B3 Laminar Flow Biological Safety Cabinet;

Shel Lab, Cornelius, OR). Tubes were then given 0.25 mL of 10^6 CFU/g of probiotic or probiotic medium (0.25 mL each for the *Lactobacillus pentosus* and *Selenomonas ruminantium* medium was combined in respective tubes to give final volume of 0.5 mL, and 0.25 mL of *Enterococcus faecium* medium was added to the *E. faecium* treatments to give final volume of 0.5 mL) by inserting a 3-mL gas-tight syringe with 27-gauge needle into the in vitro tubes containing rumen fluid. Tubes were subsequently incubated in a shaker at 39°C.

Gas production was measured, and liquid was sampled for VFA at 0, 4, 16, 24, and 48 hours. Gas production was measured in the attached syringes at 37 °C and discarded at each sampling. Gas volume was converted from mL to μmol by dividing the average gas produced for each treatment by 25,600 mL/μmol in accordance with the ideal gas law.

Liquid samples for volatile fatty acids (VFAs) were collected by inverting the in vitro tube, allowing the substrate to settle then using a 27-gauge needle and 5-mL gas tight syringe to withdraw 1 mL of sample. The sample was expelled into a 1.5 mL microcentrifuge tube and frozen in a -20°C freezer for subsequent analysis. VFA samples were prepared for GC analysis using a modified method (Erwin et al., 1961). The 1.5 mL microcentrifuge tubes containing the VFA samples were thawed at room temperature then spun in a centrifuge at 12,000 x g for 30 minutes. Supernatant (0.7 mL) was removed and phosphoric acid (0.3 mL, 10% H_3PO_4) added (De-La Rubia et al., 2009). The gas chromatograph (Hewlett-Packard model 6890) with a 4.6 m length x 0.318 cm outer diameter x 2.1 mm inner diameter packed GC column (60/80 Carboxen-1000 support, model 1-2390, Supelco, Inc, Bellefonte, PA), and flame ionization detector (FID). The split ratio of the injector port (220°C) was 100:1. Helium was used as a carrier gas with a flow

of 40 mL/min. The initial column temperature was 130°C held for 10 min, then increased to 200 °C (ramp of 80°C/min) for 1 min, and a post-run temperature of 120°C. The detector temperature was 200°C with a hydrogen and air flow of 40 mL/min and 200 mL/min, respectively. VFA production is reported as the change in concentration at each interval.

Probiotic Preparation

The *Enterococcus faecium* (241) was recovered anaerobically from our lab. The medium was autoclaved at 121°C for 45 minutes in 15 mL glass in vitro tubes (Fisherbrand, Thermo Fisher Scientific, Waltham, MA) and a 2000 mL Pyrex round bottom flask fitted with rubber stopper and wire containing medium and agar. Agar plates were immediately poured from the round bottom flask into disposable petri dishes (VWR International, Radnor, PA). A single colony was picked from the lab stock of *E. faecium* and used to inoculate the broth in the glass in vitro tubes with rubber stoppers (one colony/tube) containing the autoclaved DSMZ *Selenomonas ruminantium* medium (181; DSMZ GmbH, Braunschweig, Germany) and incubated at 39°C for 24 h. Agar plates were inoculated with the broth containing the *E. faecium* (241). The agar plates were place in a 23-Qt pressure cooker (Presto, Eau Claire, WI) containing a ratio of 2:1 CO_2/H_2 and incubated overnight at 39°C. The plates were then checked and allowed to grow under the same conditions for an addition 24 h. Once growth of colonies was observed, a single colony was picked and grown for another 48 h on a new agar plate in the pressure cooker. Gram staining was conducted on a single colony to verify gram-positive *E. faecium* isolation.

Lactobacillus pentosus (DSM- No. 20314) and *Selenomonas ruminantium* (DSM-No. 2872) were ordered as freeze-dried pellets from DSMZ GmbH (Braunschweig,

Germany). The medium for each strain was autoclaved at 121°C in 15 mL glass in vitro tubes (Fisherbrand, Thermo Fisher Scientific, Waltham, MA) and a 2000 mL Pyrex round bottom flask fitted with rubber stopper and wire containing medium and agar. The medium used for resuspension of the *L. pentosus* was autoclaved MRS Broth (69966 Lactobacillus Broth acc. to De Man, Rogosa, and Sharpe, Sigma-Aldrich, St. Louis, MO). The *Selenomonas ruminantium* medium was autoclaved *Selenomonas ruminantium* medium (181; DSMZ GmbH, Braunschweig, Germany). The inoculated media were incubated overnight at 39°C. Serial dilutions were done with each bacterium and a final concentration of 10^6 CFU/mL was used for the treatments.

Statistical Analysis

Statistical analyses were conducted using JMP Pro 12 (JMP®, Version *12*. SAS Institute Inc., Cary, NC, 1989-2007). There were two mixed models used. The first model was: $Y_{ijklm} = \mu+P_i+A_j+T_k+\gamma_{ijk(l)}+\varepsilon_{ijkl(m)}$ for response variables measured over time within tubes. The second model was: $Y_{ijkl} = \mu+P_i+A_j+\gamma_{ij(k)}+\varepsilon_{ijk(l)}$ for response variables measured only once. For each model Y is the response, μ is the mean of the population, P_i is the effect of probiotic (control for *Enterococcus faecium* (Control EF), control for *Lactobacillus pentosus* (LAB)*, Selenemonas ruminantium* (lactic acid utilizer) mix (Control LS), *Enterococcus faecium* (EF), and *Lactobacillus pentosus, Selenemonas ruminantium* mix (LS); 10^6 CFU/mL), A_j is the effect of acetate, with or without 50 mM addition, T_k is time measured at 0, 4, 16, 24, and 48 h, $\gamma_{ijk(l)}$ is the random effect of the tube nested in treatment (gas and acetate combinations or gas acetate and time), and $\varepsilon_{ijkl(m)}$ is the residual effect. All interactions were included in each model and time was a continuous variable. This model

measured the effect of treatment on total gas production and VFA production over time. Contrasts for were ran for Control EF vs EF, Control LS vs LS, and EF vs LS.

RESULTS AND DISCUSSION

Effect of Probiotic

Probiotic did not affect cumulative gas production, total VFA production, or pH (Table 1). We expected that probiotic addition would result in less production of total gas (specifically CO_2) and an increase in propionate compared to control as fermentation shifted from acetate to propionate. This observation is contrary to studies that have found effects of lactic acid bacteria (LAB) on reduction of methane (Cao et al., 2011) as well as total gas production (Muck et al., 2007; Contreras-Govea et al., 2011). Probiotic increased ($P < 0.05$) gas production between 4 – 16 h (Table 2). Gas production was the highest for *Enterococcus faecium* (EF) and decreased with the addition of the *Lactobacillus pentosus Selenomonas ruminantium* (LS) mixture. This is similar to findings that reported lower gas production when using *L. pentosus* (Muck et al., 2007) as well as reduction in methane intensity (Jeyanathan et al., 2016). Addition of LS tended to increase ($P < 0.10$) propionate production between 4 – 16 h compared to controls and decreased propionate production when *E. faecium* was used. *Lactobacillus pentosus* increased in vitro production of propionate in other studies similar to our findings (Jalč et al., 2009a; Jeyanathan et al., 2016). The addition of LS significantly increased ($P < 0.05$) butyrate production between 0 – 4 h compared to the EF treatment which had the lowest production of butyrate between 0 – 4 h. Butyrate production was decreased in other studies when using *Enterococcus faecium* as an inoculant (Beauchemin et al., 2003; Jalč et al., 2009a). The acetate: butyrate

ratio of produced VFA tended to be lower ($P < 0.10$) for the LS between 0 – 4 h compared to the EF, however the EF was numerically lower between 4 – 16 h. Acetate: butyrate ratio of produced VFA was highest ($P < 0.05$) for the control EF treatment. The initial effect of the probiotic treatment on butyrate production directly affected the A: B ratio of produced VFA. There was no effect of treatment on total VFA production or A:P ratio of produced VFA despite the tendency towards increased propionate.

Effect of Acetate

Addition of sodium acetate tended to increase ($P < 0.10$) total production of propionate between 0 – 48 h (Table 3). This was expected as we hypothesized that addition of sodium acetate may shift fermentation towards another pathway potentially due to an increase in the product concentration of acetate in the system. This would have made production of acetate less thermodynamically feasible and glucose would have been converted to other end products as indicated by the Second Law of Thermodynamics (Kohn and Boston, 2000; Ungerfeld and Kohn, 2006). There was no effect of sodium acetate addition on the production of total gas, acetate, butyrate, VFA, or pH. Sodium acetate addition increased ($P < 0.05$) total gas production between 0 – 4 h, however the opposite effect was observed between 24 – 48 h in the presence of sodium acetate (Table 4). There was a tendency towards increased ($P < 0.10$) propionate production for the sodium acetate addition between 4 – 16 h. Propionate producing bacteria tend to grow best at lower pH and in the presence of starch (Russell, 1998; Moss et al., 2000). This in vitro system may have provided optimal conditions to produce propionate when inhibiting the production of acetate. Sodium acetate addition tended ($P < 0.10$) to decrease the A:B ratio of produced VFA. This may be a result of an increase in the production of butyrate and is similar to

what has been observed in a previous study (El-Gammal et al., 2017). There was no effect of sodium acetate on the production of acetate, butyrate, total VFA, and A:P ratio of produced VFA.

Effect of Treatment Interactions

There was a tendency ($P < 0.10$) for an interaction between probiotic and sodium acetate on gas production between 16 – 24 h (Fig. 1). Addition of sodium acetate had higher gas production in the EF and LS treatments compared to EF and LS without acetate. One study (Ellis et al., 2016) observed an increase in gas production when using LAB.

The A: P ratio of produced VFA between 16 – 24 h tended ($P < 0.10$) to increase with sodium acetate addition and probiotic (Fig 2.). This may be a result of the starting product concentration of acetate. Muck et al., 2007 observed a similar increased in A: P ratio in the presence of LAB. Although numerically higher with sodium acetate addition, the A: P ratio of produced VFA for the treatments containing probiotics was less than 2. This may have been driven by the observed increase in propionate due to the presence of probiotic.

Probiotic and sodium acetate significantly decreased ($P < 0.05$) A: B ratio of produced VFA for the EF treatment both with and without sodium acetate compared to the control EF (Fig. 3). The addition of sodium acetate may have inhibited production of acetate due to product build up and shifted towards butyrate (Kohn and Boston, 2000; Ungerfeld and Kohn, 2006). *Enterococcus faecium* has been shown to increase butyrate production (Jalč et al., 2009a). There was no effect of sodium acetate addition and probiotic when comparing each probiotic to itself.

Summary

This study evaluated the effect of supplementation of LAB and sodium acetate on VFA and gas production in vitro. We observed the effects of different LAB (*E. faecium*, *L. pentosus* + *S. ruminantium*), with and without the addition of 50 mM sodium acetate. We found that *L. pentosus* + *S. ruminantium* produced the lowest gas, highest propionate and butyrate as well as decreasing the A: B ratio of produced VFA over time. *Enterococcus faecium* produced the highest gas and A: B ratio of produced VFA, but the lowest propionate and butyrate. The addition of sodium acetate in the system tended to increase the production of propionate as expected.

To effectively understand the role of lactic acid bacteria on rumen fermentation in vivo, future studies should be conducted in vitro to continue to evaluate the effect on VFA and gas production. Other factors that may affect the in vitro environment may include a system that is not optimized for the growth of LAB.

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Table 6.1. Main effect of probiotics on gas and VFA production, and pH over 48 hours

	Treatment					
	Probiotic, CFU/mL[1]					
Gas Production	**Control EF**	**EF**	**Control LS**	**LS**	**SEM**	***P***
Total Gas (μmol)[2]	786	852	835	818	46.1	0.78
Total VFA Production and pH						
Acetate (mM)	39	34	37	34	2.0	0.27
Propionate (mM)	17	20	21	19	1.2	0.36
Butyrate (mM)	16	24	23	21	2.8	0.28
Total VFA (mM)	72	78	81	75	3.6	0.42
Acetate/Propionate[3] (mM/mM)	2.3	1.8	1.9	1.8	0.19	0.29
Acetate/Butyrate (mM/mM)	2.6	1.6	1.8	1.8	0.29	0.12
pH at 48 Hours[4]	5.4	5.3	5.4	5.3	0.04	0.12

[a-b] Values within a row with different superscripts are statistically different ($P < 0.05$)

[1] Concentration of probiotic per treatment was 10^6 CFU/mL. Control EF= *Enterococcus faecium* medium without inoculum, EF = *Enterococcus faecium,* Control LS = *Lactobacillus pentosus* and *Selenomonas ruminantium* medium without inoculum, LS = *Lactobacillus pentosus* and *Selenomonas ruminantium* mix

[2] Total Gas is the gas produced between 0 and 48 hours

[3] Initial Rumen Fluid Acetate: Propionate Ratio = 3.6

[4] Initial pH = 6.39

Table 6.2. Effect of probiotics on the production of gas and VFAs by time[1]

	Treatment					
	Probiotic, CFU/mL[2]					
	Control EF	EF	Control LS	LS	SEM	*P*
Gas (μmol)						
Initial Concentration (T = 0)	0	0	0	0	--	--
0-4 h	317	322	371	322	24.8	0.50
4-16 h	381	459[ac]	405	393	17.7	< 0.05
16-24 h	39	49	44	68	13.9	0.43
24-48 h	49	22	15	34	14.2	0.46
Acetate (mM)						
Initial Concentration (T = 0)	93	93	96	96	2.4	0.67
0-4 h	15	14	17	14	2.0	0.64
4-16 h	20	16	17	18	2.1	0.57
16-24 h	2.3	2.6	2.3	3.0	1.53	0.98
24-48 h	1.3	1.5	0.6	-0.7	1.47	0.62
Propionate (mM)						
Initial Concentration (T = 0)	21	21	20	21	0.5	0.44
0-4 h	5.1	4.9	6.7	6.2	0.51	0.06
4-16 h	11	10	10	9	0.8	0.54
16-24 h	0.7	2.4	1.9	2.2	0.56	0.21
24-48 h	0.7	2.7	1.9	1.6	0.79	0.36
Butyrate (mM)						
Initial Concentration (T = 0)	10	10	9	10	0.2	0.31
0-4 h	2.6	2.3	3.1[c]	3.0	0.18	< 0.05
4-16 h	8	10	11	9	1.2	0.59
16-24 h	2.6	4.8	3.8	4.3	2.30	0.16
24-48 h	2.3	6.5	5.2	4.8	1.29	0.19
Total VFA (mM)						
Initial Concentration (T = 0)	123	124	125	127	2.6	0.70
0-4 h	23	21	27	23	2.3	0.43
4-16 h	39	36	38	37	3.3	0.90
16-24 h	6	10	8	10	2.1	0.51
24-48 h	4	11	8	6	2.9	0.39
Acetate/Propionate (mM/mM)[3]						
0-4 h	3.0	2.9	2.6	2.5	0.4	0.81
4-16 h	1.9	1.6	1.7	2.0	0.21	0.53

16-24 h	-1.2	1.0	1.4	1.1	1.22	0.54
24-48 h	3.6	0.4	0.7	1.7	1.00	0.18
Acetate/Butyrate (mM/mM)						
0-4 h	5.9	6.3	5.5	4.5	0.55	0.09
4-16 h	2.6	1.7	1.7	2.0	0.24	0.09
16-24 h	0.1	0.6	0.7	0.8	0.58	0.88
24-48 h	1.3[a]	0.2	0.5	-0.4	0.36	< 0.05

[a-c] Values within a row with different superscripts are statistically different ($P < 0.05$); a = Control EF vs EF, b = Control LS vs LS, c = EF vs LS

[1]VFA production is calculated as the change is concentration between each interval

[2]Concentration of probiotic per treatment was 10^6 CFU/mL. Control EF= *Enterococcus faecium* medium without inoculum, Control LS = *Lactobacillus pentosus* and *Selenomonas ruminantium* medium without inoculum, EF = *Enterococcus faecium*, LS = *Lactobacillus pentosus* and *Selenomonas ruminantium* mix

[3] Initial Rumen Fluid Acetate: Propionate Ratio = 3.6

Table 6.3. Main effect of sodium acetate on gas production, VFA production, and pH

	Treatment			
	Acetate (mM)[1]			
Gas Production	**No**	**Yes**	**SEM**	***P***
Total Gas (μmol)[2]	833	813	33.1	0.68
Total VFA Production and pH				
Acetate (mM)	35	37	1.4	0.35
Propionate (mM)	18	21	0.9	0.07
Butyrate (mM)	20	22	2.0	0.62
Total VFA (mM)	73	79	2.6	0.13
Acetate/Propionate (mM/mM)[3]	2.0	1.8	0.13	0.36
Acetate/Butyrate (mM/mM)	2.0	1.8	0.21	0.56
pH at 48 Hours[4]	5.3	5.3	0.03	0.58

[a-b] Values within a row with different superscripts are statistically different ($P < 0.05$)
[1]Acetate treatment indicates the addition of sodium acetate (NaOAc); N: no acetate, Y: 50 mM NaOAc
[2] Total Gas is the gas produced between 0 and 48 hours
[3] Initial Rumen Fluid Acetate: Propionate Ratio = 3.6
[4] Initial pH = 6.39

Table 6.4. Effect of acetate on the production of gas and VFAs by time[1]

	Treatment			
	Acetate (mM)[2]			
	N	Y	SEM	*P*
Gas (μmol)				
Initial Concentration (T = 0)	0	0	--	--
0-4 h	353	314	17.8	0.14
4-16 h	389[b]	430[a]	12.7	< 0.05
16-24 h	44	56	10.0	0.40
24-48 h	46[a]	13[b]	10.2	< 0.05
Acetate (mM)				
Initial Concentration (T = 0)	74[b]	115[a]	1.8	< 0.01
0-4 h	15	15	1.4	0.79
4-16 h	18	17	1.5	0.67
16-24 h	1.4	3.6	1.10	0.17
24-48 h	0.1	1.3	1.47	0.43
Propionate (mM)				
Initial Concentration (T = 0)	21	21	0.3	0.75
0-4 h	5.5	6.0	0.36	0.37
4-16 h	9	11	0.6	0.06
16-24 h	1.6	1.9	0.40	0.60
24-48 h	1.8	1.7	0.57	0.92
Butyrate (mM)				
Initial Concentration (T = 0)	10	10	0.1	0.74
0-4 h	2.6	2.9	0.13	0.13
4-16 h	9	10	0.9	0.33
16-24 h	3.6	4.1	0.46	0.45
24-48 h	5.0	4.4	0.92	0.66
Total VFA (mM)				
Initial Concentration (T = 0)	104[b]	145[a]	1.9	< 0.01
0-4 h	23	24	1.7	0.93
4-16 h	37	39	2.4	0.55
16-24 h	7	10	1.5	0.17
24-48 h	6.8	7.4	2.1	0.86
Acetate/Propionate (mM/mM)[3]				
0-4 h	2.9	2.6	0.29	0.39
4-16 h	2.0	1.6	0.15	0.12

16-24 h	1.4	-0.2	0.88	0.21
24-48 h	1.3	1.9	0.72	0.60
Acetate/Butyrate (mM/mM)				
0-4 h	6.0	5.2	0.39	0.17
4-16 h	2.2	1.8	0.17	0.08
16-24 h	0.1	1.0	0.42	0.16
24-48 h	0.4	0.4	0.26	0.81

[a-b] Values within a row with different superscripts are statistically different ($P < 0.05$)
[1]VFA production is calculated as the change is concentration between each interval
[2]Acetate treatment indicates the addition of sodium acetate (NaOAc); N: no acetate, Y: 50 mM NaOAc
[3] Initial Rumen Fluid Acetate: Propionate Ratio = 3.6

Figure 6.1. The effect of probiotic (10^6 CFU/mL) and sodium acetate addition (50 mM NaOAc) on gas production between 4 and 16 h. Probiotic reported as treatment number 1 – 4: 1) control for *Enterococcus faecium* (Control EF), 2) control for *Lactobacillus pentosus* (LAB)*, Selenemonas ruminantium* (lactic acid utilizer) mix (Control LS), 3) *Enterococcus faecium* (EF), and 4) *Lactobacillus pentosus, Selenemonas ruminantium* mix (LS). Gas production tended to increase in treatments 3 and 4 with the addition of sodium acetate. The gas production averaged 29 and 39 without acetate vs 68 and 98; SE = ± 19.7 μM with acetate for the EF and LS treatments, respectively. In the control LS treatment, gas production tended to be higher without acetate (68) vs with acetate (20); SE = ± 19.7 μM. Multiple mean comparisons test was conducted using Tukey's adjustment. A trend was determined at $P < 0.10$. Values are reported as the mean ± S.E.

Figure 6.1.

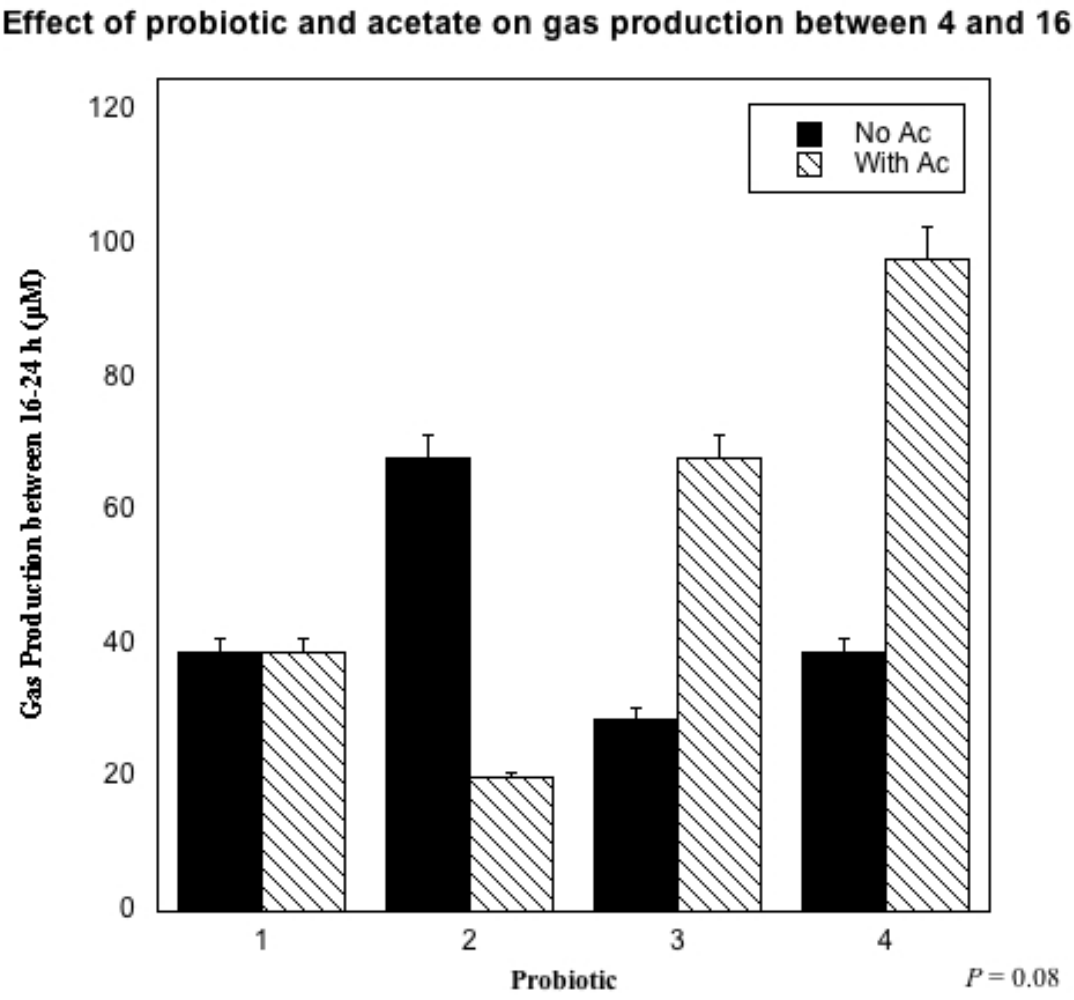

Effect of probiotic and acetate on gas production between 4 and 16 h
Gas Production between 16-24 h (µM)
120
100
80
60
40
20
0
No Ac
With Ac
1
2
3
4
Probiotic
P = 0.08

Figure 6.2. The effect of probiotic (10^6 CFU/mL) and sodium acetate addition (50 mM NaOAc) on acetate: propionate ratio between 4 and 16 h. Probiotic reported as treatment number 1 – 4: 1) control for *Enterococcus faecium* (Control EF), 2) control for *Lactobacillus pentosus* (LAB)*, Selenemonas ruminantium* (lactic acid utilizer) mix (Control LS), 3) *Enterococcus faecium* (EF), and 4) *Lactobacillus pentosus, Selenemonas ruminantium* mix (LS). Acetate: propionate ratio decreased for treatment 1 with the addition of sodium acetate. The acetate:propionate ratio averaged 3.0 without acetate vs -5.4; SE = ± 1.73 mM with acetate for the control EF. In treatments 2 – 4 acetate:propionate ratio was lower without acetate 1.3, 0.6, 0.5 vs with acetate 1.5, 1.4, 1.6; SE = ± 1.73 mM for the control LS, EF, and LS treatments, respectively Multiple mean comparisons test was conducted using Tukey's adjustment. A trend was determined at $P < 0.10$. Values are reported as the mean ± S.E.

Figure 6.2.

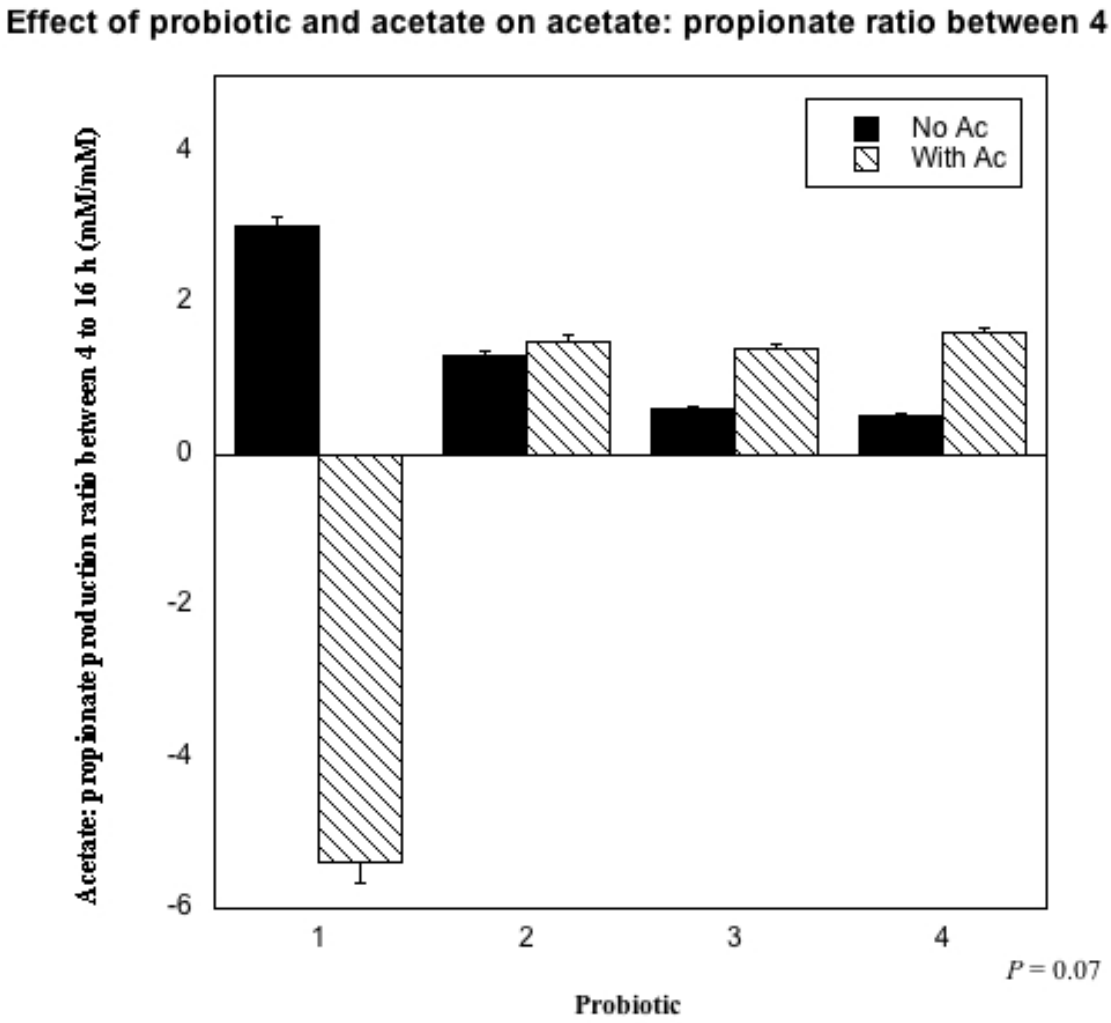
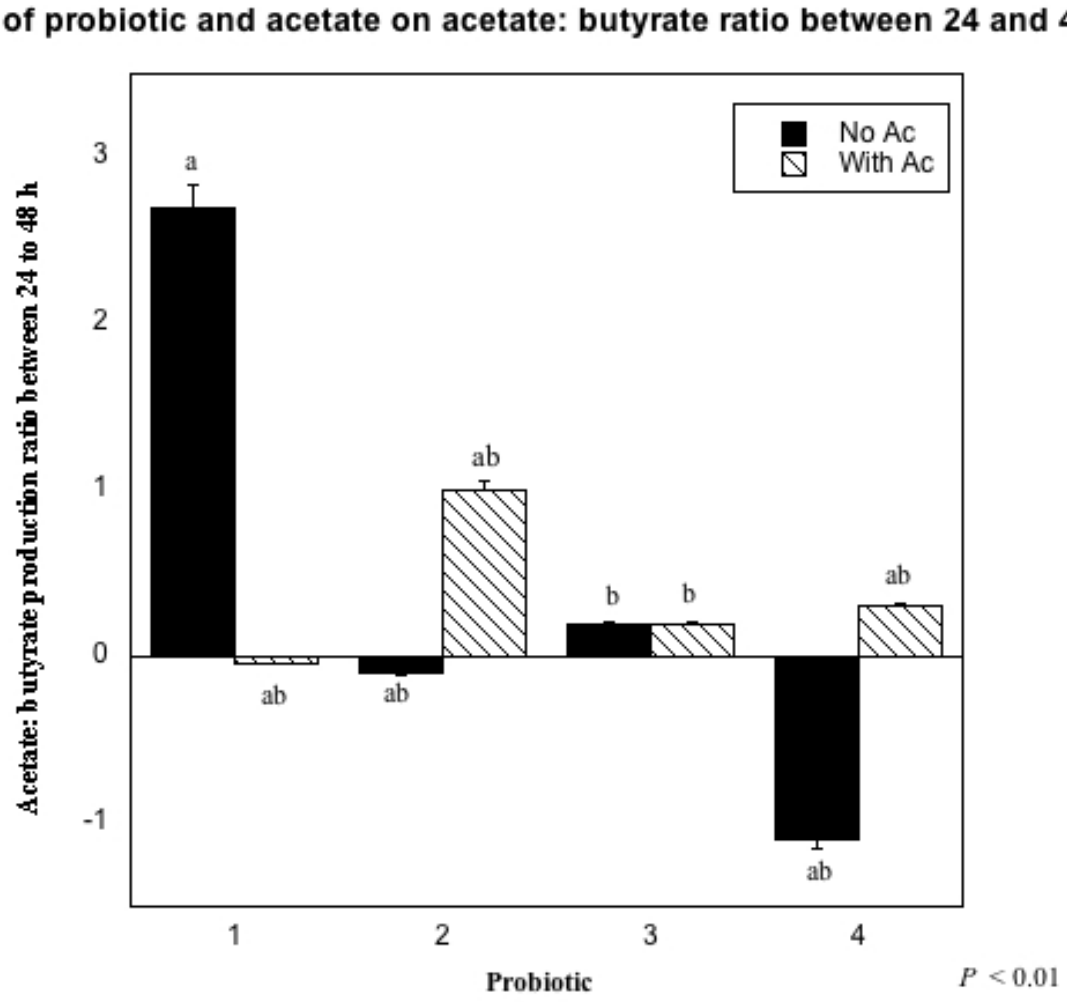

Effect of probiotic and acetate on acetate: propionate ratio between 4 and 1
No Ac
With Ac
Acetate: propionate production ratio between 4 to 16 h (mM/mM)
4
2
0
-2
-4
-6
1
2
3
4
P = 0.07
Probiotic

Figure 6.3. The effect of probiotic (10^6 CFU/mL) and sodium acetate addition (50 mM NaOAc) on acetate: butyrate ratio between 24 and 48 h. Probiotic reported as treatment number 1 – 4: 1) control for *Enterococcus faecium* (Control EF), 2) control for *Lactobacillus pentosus* (LAB)*, Selenemonas ruminantium* (lactic acid utilizer) mix (Control LS), 3) *Enterococcus faecium* (EF), and 4) *Lactobacillus pentosus, Selenemonas ruminantium* mix (LS). Acetate: butyrate ratio was higher for the control EF without acetate (2.7) vs EF without (0.18) and with acetate (0.19); SE = ± 0.51 mM. Multiple mean comparisons test was conducted using Tukey's adjustment. Significance was determined at $P < 0.05$. Values are reported as the mean ± S.E. and means with different letters (a, b, c, d) are significantly different.

Figure 6.3.


Effect of probiotic and acetate on acetate: butyrate ratio between 24 and 48 h
Acetate: butyrate production ratio between 24 to 48 h
No Ac
With Ac
a
ab
ab
ab
b
b
ab
ab
3
2
1
0
-1
1
2
3
4
Probiotic
P < 0.01

CHAPTER SEVEN

MECHANISTIC MODEL OF RUMINAL FERMENTATION INCORPORATING THERMODYNAMIC CONTROL

L. M. JUDD and R. A. KOHN

ABSTRACT: Reactions in a system are controlled by kinetics or thermodynamics. Enzyme kinetics are primarily used to represent reactions in biological systems, however thermodynamics also must be considered. Thermodynamics accounts for the concentration of products in a reaction to determine whether reactions in a system are feasible. This mechanistic model evaluates the role of thermodynamic control during rumen fermentation in the presence of lactic acid bacteria. Lactic acid bacteria produce lactate and propionate can be produced via the lactic acid pathway. This model evaluates the Gibbs free energy of key rumen reactions and their interconversions (differences) and thermodynamic efficiencies with or without different lactic acid bacteria treatments to determine whether the fermentation is being controlled by kinetics or thermodynamics, and to ascertain the sensitivity of these chemical reactions to various concentrations of glucose and hydrogen. There was little difference between the Gibbs free energy of the chemical reactions of the probiotic treatments. The thermodynamic efficiency for glucose conversion to acetate and butyrate were higher than for conversion to propionate. Thermodynamic efficiency of a reaction can imply that a reaction is closer to equilibrium (less negative ΔG), that the pathway is highly efficient (a greater ability to capture ATP energy) with complete utilization through the pathway, or that there is less potential to capture the additional energy to drive a reaction further. The thermodynamically feasible concentrations of volatile fatty acids and methane were most sensitive to hydrogen concentration in the model. Hydrogen may be a control point to target to shift fermentation of the system away from acetate towards a more favorable byproduct such as propionate or butyrate. Available glucose concentration (activity) was estimated to be about 0.3 millimolar when efficiency of conversion to volatile fatty acid was about 0.75. The model was not sensitive to glucose.

The probiotic study was limited to interpretation because several driving variables such as gas (CO_2, CH_4, H_2) had to be assumed. Therefore, the model was also evaluated using a published study (Ungerfeld et al., 2003) that measured all the gas and VFA parameters in the model except glucose. This study used increasing concentrations of acetoacetate (butyrate enhancer and electron sink) which may inhibit methane. The ΔG values for methanogenesis and acetogenesis became less negative with increasing concentration of acetoacetate, which could show decreasing energy for electron capture by those steps. There appeared to be enough free energy in the interconversions of acetate to propionate and butyrate to propionate to generate 1 ATP for energy, suggesting that 1 more ATP may be available from propionate synthesis than assumed. As glucose concentration increased, these pathways became less favorable (ΔG became more negative and more thermodynamically inefficient).

Key words: hydrogen, methane, modeling, probiotics, rumen fermentation, thermodynamics

INTRODUCTION

Ruminal fermentation leads to the degradation of starch, fiber, and protein and the subsequent production of volatile fatty acids and gases. Specifically, carbohydrates are broken down via fermentation to acetate, propionate, butyrate, methane (CH_4), and carbon dioxide (CO_2), whereas degradation of protein results in ammonia, CO_2, and other fatty acids (Russell and Hespell, 1981). The production of gases in the rumen are stoichiometrically linked with volatile fatty acid (VFA) profiles, and the pathway that produces 2 acetate, 2 CO_2 and 4 H_2 molecules per molecule of glucose is thermodynamically efficient (captures energy and drives reaction toward products) but leads to downstream synthesis of methane. Production of these byproducts from degradation of feedstuffs in the rumen has been studied, however these studies mostly focused on digestibility and nutritive quality instead of determining what controls these systems and rates of productions of these byproducts.

Greenhouse gas concentrations have become a growing concern. Methane is the second most important greenhouse gas in the United States (Kebreab et al., 2008) and around the world. About two-thirds of anthropogenic sources of methane globally are derived from agriculture (Moss et al., 2000), and about 44 percent of these global methane emissions are derived from livestock (Gerber et al., 2013). Enteric fermentation by livestock results in the production of methane and leads to a loss of up to 10% of the energy cattle ingest (Johnson and Johnson, 1995). The enteric emission of methane from cattle is a concern and requires further elucidation of the system. Inhibition of methanogenesis is one potential control point for methane emissions, and a potential means to improve production efficiency.

All chemical reactions are controlled by either kinetic and thermodynamic mechanisms or a combination thereof (Chang, 1981). Enzyme kinetic theory assumes that substrate or enzyme concentration and activity control the rate of the formation of products (Kohn, 2007). The profile of products formed depends on which are produced fastest. However, when a system is controlled by thermodynamics the rate and direction of reactions is limited by the product concentration (Kohn, 2007). The Michaelis-Menten equation is generally used to quantify kinetic parameters of biological reactions (Chang, 1981), however the rumen system does not solely follow enzyme kinetics. There is also a need to consider thermodynamics.

Lactic acid bacteria (LAB) produce and utilize lactic acid (McAllister et al., 2011). The use of LAB in vivo has been shown to increase production in dairy cows (Oetzel et al., 2007) as well as to have anti-methanogenic properties (Cao et al., 2011). *Lactobacillus pentosus* and *Enterococcus faecium* have been shown to increase production of propionate and butyrate respectively (Jalč et al., 2009a).

The purpose of this model was to potentially explain rumen fermentation using a mechanistic approach to evaluate whether these reactions are limited by thermodynamics or kinetics and to determine control points of the system that can be manipulated. The objective of this study was to model the effects of lactic acid bacteria on ruminal fermentation and to determine if supplementation of LAB shifted fermentation away from acetate towards propionate or butyrate. The model was also evaluated to test the sensitivity to glucose and hydrogen to determine their effects on ruminal fermentation.

METHODS

The Second Law of Thermodynamics

The second law of thermodynamics states that entropy (S) increases in the universe and will continue to increase over time (Chang, 1981; Engel et al., 2012). The change in Gibbs free energy (ΔG) is calculated by considering the enthalpy (heat) given off from a system when work is done (ΔH), the change in the entropy (ΔS), and the temperature (T) in Kelvin. This is depicted mathematically as: $\Delta G = \Delta H_{system} - T\Delta S_{system}$. The more negative the ΔG, the less efficient the system, which means more heat is lost and/or less available substrate is converted to product (Kohn and Boston, 2000).

Thermodynamic Efficiency

Thermodynamic efficiency is a quantification of how close a reaction is to equilibrium. This model accounts for the formation of ATP to determine the efficiencies of these reactions as the fraction of ΔG energy captured by ATP generation. For example, the ΔG for a reaction without accounting for ATP generation cannot be 0 if some Gibbs energy is needed for ATP generation. Efficiency is defined as the fraction of the ΔG remaining that is used for ATP production. The ΔG for any reaction and ATP production must be less than 0, and efficiency less than 1. Lower efficiency means that there is either more ATP for production which would result in a more negative ΔG, or that more waste (heat) was loss in the reaction.

Gibbs Free Energy Calculations

Table 7.1 depicts the free energy of formation for key rumen metabolites (Kohn and Boston, 2000). This data was used to convert the free energy of formation for key

pathways of rumen fermentation and their interconversions under standard conditions (Ungerfeld and Kohn, 2006). Table 7.2 shows the ΔG° for these reactions, and for ΔG° adjusted to ruminal temperature (311˚K). The latter values were calculated using the van't Hoff equation: $\Delta G^{\circ}_{f\ T2} = T_2/T_1\ [\ \Delta G^{\circ}_{T1} - \Delta H^{\circ}\ (T_2-T_1)/T_2]$ (Chang, 1981), where T_1 is the initial temperature (298.15 K), T_2 is the final temperature (311 K), and H°_f is the enthalpy of formation for the given reaction. Tables 7.1 and 7.2 are both calculated using book values for known metabolites. The ΔG of reactions (ΔG_{rxn}) is a calculated value based on the standard free energy change (ΔG°). The ΔG° is calculated by the difference of the sum of the reactants minus the sum of the products. Given standard conditions (1 atm pressure, 298.15˚K) and the ideal gas law gas constant R (8.314 JK/mol) the free energy of a reaction can be calculated using the following formula: $\Delta G_{rxn} = \Delta G^{\circ} + RT \ln([\text{products}]/[\text{reactants}])$, where ΔG_{rxn} is the ΔG of the reaction under standard conditions (always the same for any reaction), and ΔG° is the ΔG under standard conditions for formation of all the product from the elements minus the ΔG under standard conditions for formation of all the reactants from elements (these values are also always the same for any specific compound). The ΔG_{rxn} is based on driving variables such as the concentration of glucose, gases, or VFAs; thus ΔG_{rxn} will vary depending on the conditions. The ΔG_{rxn} can be used to determine whether reactions are near equilibrium as well as to calculate the thermodynamic efficiency of a reaction. This mechanistic model (Table 7.3) was evaluated using data from chapter 5 (the effect of lactic acid bacteria and sodium acetate on in vitro fermentation).

Thermodynamic Efficiency Calculations

Thermodynamic efficiency was evaluated for acetate and butyrate. The ΔG_{ATP} is the product of the number of ATP generated in a reaction times the ΔG of ATP. The thermodynamic efficiency is the determined by dividing $-\Delta G_{ATP}$ by ΔG.

To determine the concentration of glucose when thermodynamic efficiency is a constant (e.g. 0.75 or 1.0), the concentration had to be back calculated. For example, given the chemical reaction for the conversion of glucose to acetate $C_6H_{12}O_6 + 2\ H_2O \leftrightarrow 2\ C_2H_3O_2 + 2\ H^+ + 2CO_2 + 4\ H_2$ the ΔG_{rxn} for glucose to acetate can be calculated.

To back calculate to solve for the glucose concentration the following equation is used:

$$[\text{Products}]/\ \exp^{[(\Delta G_{ATP} - \Delta G^{\circ}_{311})/RT]} = [\text{Reactants}]$$

The final step is to solve for glucose. For glucose conversion to acetate, the left side of the equation should be divided by H_2O concentration. For glucose conversion to butyrate ($C_6H_{12}O_6 \leftrightarrow C_4H_7O_2 + H^+ + 2\ H_2 + 2\ CO_2$), the [reactant] is the concentration of glucose. The glucose sensitivity was based on the assumed concentration of glucose: 0.3 mmol/l and glucose were increased or decreased by a magnitude of 10 (0.3, 0.03, 3.0, and 30 mmol/l). Hydrogen sensitivity was evaluated in the same manner. With the assumed hydrogen pressure of 0.00152 atm, the values were increased or decreased by a magnitude of 10 to yield the following concentrations (0.00152, 0.000152, 0.0152, and 0.152 atm).

RESULTS AND DISCUSSION

Probiotic Study

The study evaluated the addition of sodium acetate and lactic acid bacteria on volatile fatty acid and gas profile. The purpose of this model was to determine the effect of the probiotic treatments on these pathways to quantify the Gibbs energy change (ΔG) of these reactions under test conditions, the thermodynamic efficiencies, and to determine whether reactions are limited by kinetics or thermodynamics. When evaluating the Gibbs free energy of rumen fluid in vitro flasks using these probiotics (control EF= *Enterococcus faecium* medium without inoculum, EF = *Enterococcus faecium,* control LS = *Lactobacillus pentosus* and *Selenomonas ruminantium* medium without inoculum, LS = *Lactobacillus pentosus* and *Selenomonas ruminantium* mix) the explanation of the results by the model are similar for each treatment (Table 7.4). The model assumed the following: [glucose] = 0.3 mmol/L, [CH_4] = 0.3 atm, [CO_2] = 0.7 atm, [H_2O] = 50 mmol/l, [H_2] = 0.00152 atm, [ATP] = 1 mmol/l, [ADP][P_i] = 0.002 mmol/l, pH = 6.55. Methane, carbon dioxide, water, hydrogen, ATP, and ADP were based on previously published assumptions (Kohn and Boston, 2000) as this study did not directly measure individual gas production. The Gibbs free energy for the interconversions of acetate to propionate and butyrate to propionate could potentially produce 0.5 ATP of energy. The model assumes that there is no energy captured during the interconversions of these, however it may be feasible to capture that energy, thus pushing the reaction further. A difference like this could show that more ATP is generated than thought for propionate synthesis, or less ATP is generated from acetate and butyrate synthesis. However, as will

be shown these reactions are also sensitive to H_2 concentrations, which were based on previous studies and not measured in this study. Butyrate to acetate conversion is near 0, which indicates that this reaction is close to equilibrium and glucose conversion to acetate or butyrate with equivalent ATP production is likely. The pH was based on the pH measured in the experiment. The values of acetate, propionate, and butyrate from table 7.3 were used for each respective treatment. This model showed that the formation of methane under these conditions was the most thermodynamically efficient (complete utilization of the pathway) for the use of free H_2 and the results of the model favored the production of acetate and butyrate over propionate. This observation is like that of Kohn and Boston, 2000. This is unexpected as the LAB shifted fermentation towards propionate production in the study, so we expected that the pathway for glucose to propionate would be more efficient and the probiotic would make ΔG more negative providing more energy to drive the reaction. However, because we didn't account for changes in gases due to the treatments, the effect on ΔG for methanogenesis or reductive acetogenesis may have been missed. *Enterococcus faecium* increased ΔG for butyrate relative to acetate and propionate thus making this pathway slightly more efficient. Both *E. faecium* and *L. pentosus* increased ΔG for the acetate to propionate pathway making it slightly more efficient. There wasn't much difference in the ΔG of the probiotics for the conversion of glucose to propionate. However, the ΔG for glucose to propionate was more negative than glucose to acetate or glucose to butyrate and could potentially drive this reaction further though this would not increase the efficiency of the pathway. If the glucose to propionate pathway was able to generate more ATP for energy (5 instead of 4) the efficiency of this pathway would increase. The increase in propionate production by

the probiotic treatment is interesting as most likely there was more hydrogen in the in vitro system which leads to the increased propionate production. If less hydrogen was available for methane synthesis, this could potentially explain why the ΔG of methanogenesis was less negative and appeared to be more efficient. The presence of sodium acetate (50 mM) in the treatments was utilized to inhibit production of acetate, as we believed addition of sodium acetate will result in the pathway being thermodynamically limited, however in the study there was no difference in the production of acetate with or without sodium acetate addition. An inhibitor such as sodium acetate should have made the pathway of glucose to acetate less efficient and the ΔG should have been more positive, but this was not observed with any of the treatments.

Thermodynamic Efficiency and Glucose Sensitivity

The efficiency and effectiveness of glucose were unknown. Therefore, the results of the model were used to what the glucose concentration would be for maximal efficiency of these reactions. The model was tested to determine the effect of probiotics on the thermodynamic efficiency of butyrate (Table 7.5) was 1 ($\Delta G_{rxn} = 0$). The efficiencies for methanogenesis and acetogenesis were decreased only for the *Enterococcus faecium* treatment (Table 7.5). The decrease in efficiency is explained by the result of the ΔG for methanogenesis and ΔG for acetogenesis both becoming positive indicating this reaction is no longer thermodynamically feasible under these conditions. When controlling the efficiency of butyrate (Table 7.5), the efficiencies increased for both glucose to acetate and glucose to propionate and there was a similar effect on the ΔG for methanogenesis and the ΔG acetogenesis as observed by controlling for the efficiency of acetate, when glucose to butyrate $\Delta G_{rxn} = 0$. The efficiency shows that more ATP can be made from the glucose to

propionate pathway compared to glucose to acetate or glucose to propionate. Neither the pathway for methanogenesis or acetogenesis was feasible under these conditions. There is potential to make more ATP from acetogenesis and methanogenesis. The effect of *Enterococcus faecium* on methanogenesis and acetogenesis is not unexpected as the *E. faecium* bug that was isolated was shown in previous studies to be efficient at reductive acetogenesis (Kohn and Kim, 2015). Most interesting is that to achieve these thermodynamic efficiencies, each model required very low (physically impossible) to measure concentrations of glucose and averaged 5.24×10^{-15} mmol/l (acetate) and 1.84×10^{-14} mmol/l (butyrate). The concentration of glucose when setting the maximal efficiency to produce acetate was: 5.15×10^{-15}, 5.17×10^{-15}, 5.40×10^{-15}, and 5.25×10^{-15} (mmol/l) for the control EF, EF, control LS, and LS treatments respectively. The same result of low concentrations of glucose from the model when controlling for the maximal efficiency to produce butyrate and was: 1.56×10^{-14}, 2.10×10^{-14}, 1.81×10^{-14}, and 1.88×10^{-14} (mmol/l) for the control EF, EF, control LS, and LS treatments respectively. Except for the EF treatment the model did not have different results. This glucose concentration is quite different than that which we assumed. This low concentration of glucose is consistent with other studies as only the most efficient microbes can use low concentrations of glucose due to the competition for glucose by microorganisms during fermentation (Kohn and Kim, 2015). Under the conditions where the thermodynamic efficiencies of the reaction were at maximal efficiency (about 0.75) for conversion to VFA), the pathway for acetogenesis and methanogenesis became more thermodynamically favorable for *E. faecium.* To evaluate whether the probiotics had reached maximal efficiency, a glucose sensitivity analysis for *Enterococcus faecium* was conducted using glucose concentrations of 0.03, 0.3, 3.0, and

30 mmol/l (Table 7.6). As observed in Table 7.5 and 7.6, the increase in glucose concentration decreased the overall efficiency of acetate, propionate, and butyrate; however, the model was not very sensitive to the changes in glucose and the ratio of VFA doesn't change as glucose increases. This could indicate that these reactions are thermodynamically limited as the microorganisms that utilize glucose are not very efficient with the higher concentrations of glucose. The ΔG for both methanogenesis and acetogenesis were less negative and more efficient as observed in Table 7.4 which is not unexpected as glucose is not a part of the chemical reactions of methanogenesis or acetogenesis.

Inhibition of methane can occur by redirecting produced hydrogen to a different pathway such as the formation of propionate to reduce the availability of hydrogen for the synthesis of methane. If this were to occur, the pathway for methanogenesis would become less efficient (ΔG more positive) and the pathway for propionate would become more thermodynamically efficient (ΔG less negative). Lactic acid bacteria may potentially compete with acetate producing bacteria for glucose or for hydrogen and potentially reduce conversion of CO_2 and H_2 to methane. Lactic acid bacteria produce lactic acid and lactic acid can be converted to propionate (McAllister et al., 2011). Increased hydrogen pressure and the presence of propionate or butyrate intermediates have been shown to increase the production of both propionate and butyrate, however they also tend to increase acetate production (Ungerfeld and Kohn, 2006). One evaluation of this model was to determine the sensitivity of the probiotic treatments in the model to differing concentrations of hydrogen. Table 7.8 shows the effect of increasing hydrogen concentration on the thermodynamic efficiencies of key reactions for the EF treatment. Except for the

conversion of glucose to propionate, as the concentration of hydrogen increased the thermodynamic efficiencies increased. As the assumed concentration of hydrogen was increased in the model, the ΔG for glucose to propionate became more negative indicating this reaction was more thermodynamically feasible and could continue to go forward. The major limitation in the probiotic study is that hydrogen was not directly measured. This could lead to an issue of underestimating the efficiencies of these probiotics as the only driving variable that changed was the VFA concentrations. As the actual concentration of hydrogen is unknown it is unclear as to whether these probiotics increased the efficiencies of acetate or butyrate, or whether the probiotics made the synthesis of propionate from glucose more thermodynamically favorable. This is important as the results of the model indicate that the Gibbs free energy of the reactions are sensitive to hydrogen concentrations. The glucose to propionate reaction had decreased efficiency as the concentration of hydrogen increased. This was interesting as production of propionate has been shown to be a hydrogen sink and direct competitor for hydrogen to methanogenesis. The performance of the model follows that of Ungerfeld and Kohn (2006) which demonstrated that ΔG for key ruminal reactions decrease as hydrogen pressure increases. This is also interesting as the EF treatment tended to increase production of propionate, however this potential increase in production is not observed in the ΔG_{rxn}. One possible explanation is that lactic acid bacteria produce lactic acid, which can then synthesize propionate via the lactic acid pathway. The observed tendency towards increased propionate production may be a result of the lactic acid pathway producing propionate, which the model would not account for in the synthesis of glucose to propionate. The lack of individual gas measurements in the probiotic study limits the interpretation of the model

for the effect of the probiotics. One way to better develop this study and therefore the model results and interpretation would be to measure not only total gas production, but hydrogen, carbon dioxide, and methane as well. This will allow individual values of these concentrations to be used as driving variables which will result in different ΔG_{rxn} and efficiencies. Due to not having these measured variables, the ΔG_{rxn} and efficiencies are most likely similar as the only changes were to the VFA concentrations which were not statistically different in the study.

Ungerfeld 2003

As stated previously, one of the limitations of this model was that the gases were not individually measured, therefore these values had to be assumed. To test the model further, this model was evaluated using data from another study (Ungerfeld et al., 2003) to determine its overall performance. The purpose of this study was to evaluate alternative electron sinks and their ability to inhibit methanogenesis. Acetoacetate is a butyrate enhancer and was used as a potential electron sink and differing concentrations were used by Ungerfeld et al., 2003 to determine the effect on methanogenesis. Table 7.8 shows the Gibbs free energy of the reactions. In this model, the thermodynamic efficiencies of methanogenesis and acetogenesis were both much lower than the probiotic study. The ΔG for glucose to butyrate also became less negative and more efficient as the concentration of acetoacetate was increased. This would indicate the acetoacetate treatment was effective in increasing the synthesis of butyrate. The ΔG for the interconversion of acetate to propionate and butyrate to propionate become less negative as the concentration of acetoacetate increased. These ΔG of these interconversions indicate that it is possible to generate 1 ATP for energy. If this occurred, these pathways would become more efficient.

Conversely, as the concentration of acetoacetate increased the ΔG_{rxn} became less negative for methanogenesis and acetogenesis. At the highest concentration of acetoacetate, the thermodynamic efficiency for methanogenesis and acetogenesis also increased. The efficiency of both pathways was very low, indicating that the addition of acetoacetate was inhibiting those pathways. If the pathways for methanogenesis and acetogenesis could produce more ATP for energy (i.e. methanogenesis: 1.5 mol vs 1 mol and acetogenesis: 1 mol vs 0.2 mol) the efficiencies of these pathways would increase.

The Ungerfeld model also required an assumption to be made for glucose, therefore a sensitivity analysis controlling for the concentration of glucose was conducted. The efficiencies of the reactions decreased with increasing concentrations of glucose (Table 7.9). These ΔG_{rxn} became more negative as glucose concentration increased potentially indicating that these reactions were becoming more favorable. The efficiencies of glucose to acetate, glucose to propionate, and glucose to butyrate decreased as glucose concentrations were increased. The strength of this study compared to the probiotic study is that there are measurements for individual gases (carbon dioxide, methane, and hydrogen). Acetoacetate is an electron sink, yet as hydrogen and glucose concentrations increased the efficacy of these reactions decreased making these reactions more favorable. As expected the butyrate enhancer did inhibit methanogenesis. The ΔG of methanogenesis in less negative indicating this reaction is near equilibrium and that no more energy can be captured from this pathway.

Studies have shown that methanogenesis can be inhibited by redirecting hydrogen (Chalupa, 1977). Although, enzyme kinetics plays a role in rumen fermentation, the effect of thermodynamics on the system cannot be ignored (Kohn, 2014). Most fermentation

systems have been shown to be near thermodynamic equilibrium (Kohn and Kim, 2015) and studies have shown that these pathways are close to equilibrium (Kohn and Boston, 2000).

Regarding both studies, ruminal fermentation was limited by both kinetics and thermodynamics. The effect of differing glucose concentrations demonstrated that the substrate concentrations can prevent a reaction from proceeding forward and thus will reach its maximal efficiency with higher levels of glucose. Though the efficiencies of these reactions in both studies decreased with increased concentration of glucose, the efficiencies did not greatly differ from that of the original assumed glucose concentration. In the probiotic study, sodium acetate (50 mM) was supplemented to aid the LAB by potentially inhibiting the production of acetate, thus allowing the pathway to shift. However, the addition of acetate did not affect the production of acetate, thus considering the end products, the pathway to synthesize acetate was still thermodynamically feasible (more negative ΔG) under the in vitro conditions. Propionate production tended to increase, though the observed in the ΔG_{rxn} (glucose to propionate) was more negative thus indicating the pathway was thermodynamically favorable for the probiotic treatment though less efficient than anticipated. The tendency for an increase in propionate production could be a result of the lactic acid pathway or the succinate pathway which also synthesizes propionate. Furthermore, when evaluating the sensitivity to higher concentrations of hydrogen, the thermodynamic efficiencies slightly increased for the synthesis of acetate and butyrate but decreased for propionate. These observed efficiencies in the probiotic study are most likely near their maximal efficiencies and thus potentially controlled by thermodynamics.

Summary

The purpose of the model was to evaluate the effect of direct fed microbials (specifically lactic acid bacteria) on ruminal fermentation, to determine whether these reactions were sensitive to any driving variables such as glucose or hydrogen, and to elucidate whether these systems are being controlled by kinetics or thermodynamics. Direct fed microbials are used as supplements in dairy cows to improve performance parameters, such as milk production. This model results indicate that these different lactic acid bacteria had similar free energy of reactions and thermodynamic efficiency regardless of treatment. This finding could indicate that we most likely couldn't detect a difference due to improved efficiency of any particular reaction. *Enterococcus faecium*, was the only bacteria in which acetogenesis and methanogenesis were more thermodynamically favorable once these ruminal fermentation reactions had reached maximal efficiency. When evaluating the sensitivity of glucose in the model, the studies used demonstrate that for a product to be at equilibrium, there is a requirement for very low concentrations of free glucose. The interpretation of the probiotic study is limited due to lacking individual gas concentrations. The Ungerfeld (2003) study was sensitive to glucose as well as to hydrogen and reactions approached equilibrium at biologically relevant concentrations of glucose. Glucose concentration may be a kinetically limiting factor in these reactions, however the system is thermodynamically limited as the production of end products has most likely reached maximal efficiency. Controlling for hydrogen may be the key intervention to shift fermentation from acetate and subsequent downstream synthesis of methane towards a more favorable product. The use of both

enzyme kinetics and thermodynamics when modeling biological systems will enhance understanding of the rumen fermentation system. Future studies of this model could evaluate the impact of inhibitors of methanogenesis through conducting a meta-analysis where individual gas measurements are made in addition to rumen production parameters.

Table 7.1 Free energy of formation (ΔG_f) and enthalpy of formation (kJ/mol) of rumen metabolites[1].

Metabolite	ΔG°_{298}	ΔH°_{f}
α, β-D-Glucose (aq) ($C_6H_{12}O_6$)	-916.97	-1263.78
Acetate (aq)	-376.89	-485.6
Propionate (aq)	-373.82	-511.7
Butyrate (aq)	-372.04	-533.55
Lactate (aq)	-516.72	-686.64
Methane (aq)	-50.79	-74.85
Carbon dioxide (aq)	-386.23	-412.92
Water (l)	-237.19	-285.84
Hydrogen (g)	0	0

[1]Data adapted from (Kohn and Boston, 2000) and are book values as published in Chang, 1981.

Table 7.2 Conversion of free energy of formation for selected reactions to ruminal conditions.

Reaction	Formula	ΔG°_{298}	ΔH°	ΔG°_{311}
Glucose to acetate	$C_6H_{12}O_6 + 2\ H_2O \leftrightarrow 2\ C_2H_3O_2 + 2\ H^+ + 2CO_2 + 4\ H_2$	-134.89	38.4	-142.45
Glucose to propionate	$C_6H_{12}O_6 + 2\ H_2 \leftrightarrow 2\ C_3H_5O_2 + 2\ H^+ + 2\ H_2O$	-305.05	-331.3	-303.90
Glucose to butyrate	$C_6H_{12}O_6 \leftrightarrow C_4H_7O_2 + H^+ + 2\ H_2 + 2\ CO_2$	-227.53	-97.6	-233.20
Acetate to propionate	$2\ C_2H_3O_2 + 2\ CO_2 + 6\ H_2 \leftrightarrow 2\ CH_3H_5O_2 + 4\ H_2O$	-170.16	-369.7	-161.46
Acetate to butyrate	$2\ C_2H_3O_2 + H^+ + 2\ H_2 \leftrightarrow C_4H_7O_2 + 2\ H_2O$	-92.64	-136	-90.70
Propionate to butyrate	$2\ C_3H_5O_2 + H^+ + 2\ H_2O \leftrightarrow C_4H_7O_2 + 2\ CO_2 + 4\ H_2$	77.52	233.7	70.71
Methanogenesis	$CO_2 + 4\ H_2 \leftrightarrow CH_4 + 2\ H_2O$	-138.94	-233.6	-134.81
Acetogenesis	$2\ CO_2 + 4\ H_2 \leftrightarrow C_2H_3O_2 + H^+ + 2\ H_2O$	-78.81	-231.4	-72.15
ADP to ATP	$ADP + P_i + H^+ \leftrightarrow ATP + H_2O$	-9	24.3	-10.453

Values were calculated from data in Table 6.1, ATP values were acquired from (Rekharsky et al., 1986) as published by (Kohn and Boston, 2000).

Table 7.3 Final VFA concentrations of probiotic treatments.

	Probiotic			
Volatile Fatty Acid Concentration (mmol/l)	**Control EF**	**EF**	**Control LS**	**LS**
Acetate	94.3	94.5	96.6	95.3
Propionate	21.7	23.7	21.9	22.6
Butyrate	12.3	16.5	14.2	14.8

Data acquired from Chapter 5: The effect of lactic acid bacteria and sodium acetate on in vitro fermentation. Probiotic treatments were: Control EF= *Enterococcus faecium* medium without inoculum, EF = *Enterococcus faecium,* Control LS = *Lactobacillus pentosus* and *Selenomonas ruminantium* medium without inoculum, LS = *Lactobacillus pentosus* and *Selenomonas ruminantium* mix

Table 7.4 Free energy (kJ/mol substrate) and efficiency (kJ/kJ) of rumen fermentation reactions for probiotic treatments.

Reaction	Formula	No. ATP	ΔG_{rxn}				$-\Delta G_{ATP}/\Delta G_{rxn}$					
			Control EF	EF	Control LS	LS	Control EF	EF	Control LS	LS	SEM	*P*
Glucose to acetate	$C_6H_{12}O_6$+ 2 H_2O ↔ 2 $C_2H_3O_2$ + 2 H^+ + 2CO_2 + 4 H_2	4	-300.87	-300.86	-300.74	-300.81	0.73	0.73	0.73	0.73	0.126	0.71
Glucose to propionate	$C_6H_{12}O_6$ + 2 H_2 ↔ 2 $C_3H_5O_2$ + 2 H^+ +2 H_2O	4	-326.95	-326.49	-326.90	-326.74	0.67	0.67	0.67	0.67	0.137	0.28
Glucose to butyrate	$C_6H_{12}O_6$ ↔ $C_4H_7O_2$ + H^+ +2 H_2 + 2 CO_2	4	-297.99	-297.23	-297.62	-297.52	0.73	0.74	0.74	0.74	0.232	0.24
Acetate to propionate	2 $C_2H_3O_2$ + 2CO_2+ 6 H_2 ↔ 2 $CH_3H_5O_2$ + 4 H_2O	0	-26.08	-25.63	-26.15	-25.92	0	0	0	0	0.189	0.30
Acetate to butyrate	2 $C_2H_3O_2$ + H^+ + 2 H_2 ↔ $C_4H_7O_2$ + 2 H_2O	0	2.87	3.62	3.12	3.30	0	0	0	0	0.297	0.29
Propionate to butyrate	2 $C_3H_5O_2$ + H^+ + 2 H_2O ↔ $C_4H_7O_2$ +2 CO_2 + 4 H_2	0	28.95	29.25	29.28	29.22	0	0	0	0	0.114	0.30
Methanogenesis	CO2 + 4 H2 ↔ CH4 + 2 H2O	1	-49.66	-49.66	-49.66	-49.66	1.1	1.1	1.1	1.1	--	--
Acetogenesis	2 CO2 + 4 H2 ↔ C2H3O2 + H+ + 2 H2O	0.2	-28.07	-28.06	-28	-28.04	0.39	0.39	0.39	0.39	0.063	0.71
ADP to ATP	ADP+ P_i + H^+ ↔ ATP +H_2O	1	54.73	54.73	54.73	54.73	--	--	--	--	--	--

Data are calculated from Table 6.2 and assuming ruminal conditions from Table 6.3 as well as the following: [glucose] = 0.3 mmol/l, [CH_4] = 0.3 atm, [CO_2] = 0.70 atm, [H_2O] = 50 mmol/l, [H_2] = 0.00152 atm, [ATP] = 1 mmol/l, [ADP][P_i] = 0.002 mmol/l, pH = 6.55

Table 7.5. Free energy (kJ/mol substrate) and efficiency (kJ/kJ) of rumen fermentation reactions when controlling for thermodynamic efficiency of butyrate.

			ΔG_{rxn}				$-\Delta G_{ATP}/\Delta G_{rxn}$			
Reaction	**Formula**	**No. ATP**	**Control EF**	**EF**	**Control LS**	**LS**	**Control EF**	**EF**	**Control LS**	**LS**
Glucose to acetate	$C_6H_{12}O_6 + 2\ H_2O \leftrightarrow 2\ C_2H_3O_2 + 2\ H^+ + 2CO_2 + 4\ H_2$	4	-221.79	-222.53	-222.03	-222.21	0.99	0.98	0.99	0.99
Glucose to propionate	$C_6H_{12}O_6 + 2\ H_2 \leftrightarrow 2\ C_3H_5O_2 + 2\ H^+ + 2\ H_2O$	4	-247.86	-248.17	-248.19	-248.13	0.88	0.88	0.88	0.88
Glucose to butyrate	$C_6H_{12}O_6 \leftrightarrow C_4H_7O_2 + H^+ + 2\ H_2 + 2\ CO_2$	4	-218.91	-218.91	-218.91	-218.91	1	1	1	1
Acetate to propionate	$2\ C_2H_3O_2 + 2CO_2 + 6\ H_2 \leftrightarrow 2\ CH_3H_5O_2 + 4\ H_2O$	0	-26.08	-25.63	-26.15	-25.92	0	0	0	0
Acetate to butyrate	$2\ C_2H_3O_2 + H^+ + 2\ H_2 \leftrightarrow C_4H_7O_2 + 2\ H_2O$	0	2.87	3.62	3.12	3.30	0	0	0	0
Propionate to butyrate	$2\ C_3H_5O_2 + H^+ + 2\ H_2O \leftrightarrow C_4H_7O_2 + 2\ CO_2 + 4\ H_2$	0	28.95	29.25	29.28	29.22	0	0	0	0
Methanogenesis	$CO2 + 4\ H2 \leftrightarrow CH4 + 2\ H2O$	1	-49.66	-49.66	-49.66	-49.66	1.10	-2.62	1.10	1.10
Acetogenesis	$2\ CO2 + 4\ H2 \leftrightarrow C2H3O2 + H+ + 2\ H2O$	0.2	-28.07	-28.06	-28.00	-28.04	0.39	-0.25	0.39	0.39
ADP to ATP	$ADP + P_i + H^+ \leftrightarrow ATP + H_2O$	1	54.73	54.73	54.73	54.73	--	--	--	--

Data are calculated from Table 6.2 and assuming ruminal conditions from Table 6.3 as well as the following: $[CH_4] = 0.3$ atm, $[CO_2] = 0.7$ atm $[H_20] = 50$ mmol/l, $[H_2] = 0.00152$ atm, [ATP] = 1 mmol/l, $[ADP][P_i] = 0.002$ mmol/l, pH = 6.55.

Table 7.6. Free energy (kJ/mol substrate) and efficiency (kJ/kJ) of rumen fermentation reactions when controlling for the concentration of glucose[1].

			ΔG_{rxn}				$-\Delta G_{ATP}/\Delta G_{rxn}$			
Reaction	**Formula**	**No. ATP**	**[GLC][2] = 0.03**	**[GLC] = 0.3**	**[GLC] = 3.0**	**[GLC] = 30**	**[GLC] = 0.03**	**[GLC] = 0.3**	**[GLC] = 3.0**	**[GLC] = 30**
Glucose to acetate	$C_6H_{12}O_6 + 2\ H_2O \leftrightarrow 2\ C_2H_3O_2 + 2\ H^+ + 2CO_2 + 4\ H_2$	4	-294.90	-300.86	-306.81	-312.76	0.74	0.73	0.71	0.70
Glucose to propionate	$C_6H_{12}O_6 + 2\ H_2 \leftrightarrow 2\ C_3H_5O_2 + 2\ H^+ + 2\ H_2O$	4	-320.54	-326.49	-332.44	-338.40	0.68	0.67	0.66	0.65
Glucose to butyrate	$C_6H_{12}O_6 \leftrightarrow C_4H_7O_2 + H^+ + 2\ H_2 + 2\ CO_2$	4	-291.28	-297.23	-303.19	-309.14	0.75	0.74	0.72	0.71
Acetate to propionate	$2\ C_2H_3O_2 + 2CO_2 + 6\ H_2 \leftrightarrow 2\ CH_3H_5O_2 + 4\ H_2O$	0	-25.63	-25.63	-25.63	-25.63	0	0	0	0
Acetate to butyrate	$2\ C_2H_3O_2 + H^+ + 2\ H_2 \leftrightarrow C_4H_7O_2 + 2\ H_2O$	0	3.62	3.62	3.62	3.62	0	0	0	0
Propionate to butyrate	$2\ C_3H_5O_2 + H^+ + 2\ H_2O \leftrightarrow C_4H_7O_2 + 2\ CO_2 + 4\ H_2$	0	29.25	29.25	29.25	29.25	0	0	0	0
Methanogenesis	CO2 + 4 H2 ↔ CH4 + 2 H2O	1	-49.66	-49.66	-49.66	-49.66	1.10	1.10	1.10	1.10
Acetogenesis	$2\ CO_2 + 4\ H_2 \leftrightarrow C_2H_3O_2 + H^+ + 2\ H_2O$	0.2	-28.06	-28.06	-28.06	-28.06	0.39	0.39	0.39	0.39
ADP to ATP	$ADP + P_i + H^+ \leftrightarrow ATP + H_2O$	1	54.73	54.73	54.73	54.73	--	--	--	--

[1]Data are calculated from Table 6.2 and assuming ruminal conditions from Table 6.3 as well as the following: $[CH_4] = 0.3$ atm, $[CO_2] = 0.7$ atm $[H_20] = 50$ mmol/l, $[H_2] = 0.00152$ atm, [ATP] = 1 mmol/l, $[ADP][P_i] = 0.002$ mmol/l, pH = 6.55.
[2]Model was run with the following [glucose] = 0.03, 0.3, 3.0, and 30 mmol/l

Table 7.7. Free energy (kJ/mol substrate) and efficiency (kJ/kJ) of rumen fermentation reactions when controlling for sensitivity of $[H_2]$.

			ΔG_{rxn}				$-\Delta G_{ATP}/\Delta G_{rxn}$			
Reaction	**Formula**	**No. ATP**	**$[H_2]^2$ = 0.000152**	**$[H_2]$ = 0.00152**	**$[H_2]$ = 0.0152**	**$[H_2]$ = 0.152**	**$[H_2]$ = 0.000152**	**$[H_2]$ = 0.00152**	**$[H_2]$ = 0.0152**	**$[H_2]$ = 0.152**
Glucose to acetate	$C_6H_{12}O_6$+ 2 H_2O↔ 2 $C_2H_3O_2$ + 2 H^+ + 2CO_2+ 4 H_2	4	-324.67	-300.86	-277.04	-253.23	0.67	0.73	0.79	0.86
Glucose to propionate	$C_6H_{12}O_6$ + 2 H_2 ↔ 2 $C_3H_5O_2$ + 2 H^+ +2 H_2O	4	-314.58	-326.49	-338.40	-350.30	0.70	0.67	0.65	0.62
Glucose to butyrate	$C_6H_{12}O_6$ ↔ $C_4H_7O_2$ + H^+ +2 H_2 + 2 CO_2	4	-309.14	-297.23	-285.33	-273.42	0.71	0.74	0.77	0.80
Acetate to propionate	2 $C_2H_3O_2$ + 2CO_2+ 6 H_2↔ 2 $CH_3H_5O_2$ + 4 H_2O	0	10.09	-25.63	-61.35	-97.08	0	0	0	0
Acetate to butyrate	2 $C_2H_3O_2$ + H^+ + 2 H_2 ↔ $C_4H_7O_2$ + 2 H_2O	0	15.53	3.62	-8.28	-20.19	0	0	0	0
Propionate to butyrate	2 $C_3H_5O_2$ + H^+ + 2 H_2O ↔ $C_4H_7O_2$ +2 CO_2 + 4 H_2	0	5.44	29.25	53.07	76.88	0	0	0	0
Methanogenesis	CO2 + 4 H2 ↔ CH4 + 2 H2O	1	-25.84	-49.66	-73.47	-97.29	2.12	1.10	0.74	0.56
Acetogenesis	2 CO2 + 4 H2 ↔ C2H3O2 + H+ + 2 H2O	0.2	-4.25	-28.06	-51.88	-75.69	2.58	0.39	0.21	0.14
ADP to ATP	ADP+ P_i + H^+ ↔ ATP +H_2O	1	54.73	54.73	54.73	54.73	--	--	--	--

[1]Data are calculated from Table 6.2 and assuming ruminal conditions from Table 6.3 for example *E. faecium* treatment, as well as the following: [glucose] = 0.3 mmol/l, $[CH_4]$ = 0.3 atm, $[CO_2]$ = 0.70 atm, $[H_2O]$ = 50 mmol/l, [ATP] = 1 mmol/l, [ADP][P_i] = 0.002 mmol/l, pH = 6.55. Hydrogen concentrations are atm

[2]Model was run with the following $[H^2]$ = 0.000152, 0.00152, 0.0152, 0.152 atm.

Table 7.8. Free energy (kJ/mol substrate) and efficiency (kJ/kJ) of rumen fermentation reactions for different levels of acetoacetate treatment. Adapted from Ungerfeld et al., 2003[1].

			ΔG_{rxn}				$-\Delta G_{ATP}/\Delta G_{rxn}$			
			Acetoacetate (mM)							
Reaction	**Formula**	**No. ATP**	**0[2]**	**6**	**12**	**18**	**0**	**6**	**12**	**18**
Glucose to acetate	$C_6H_{12}O_6 + 2\ H_2O \leftrightarrow 2\ C_2H_3O_2 + 2\ H^+ + 2CO_2 + 4\ H_2$	4	-296.82	-297.26	-291.25	-293.58	0.76	0.78	0.78	0.77
Glucose to propionate	$C_6H_{12}O_6 + 2\ H_2 \leftrightarrow 2\ C_3H_5O_2 + 2\ H^+ + 2\ H_2O$	4	-367.46	-367.01	-366.63	-366.20	0.62	0.62	0.62	0.62
Glucose to butyrate	$C_6H_{12}O_6 \leftrightarrow C_4H_7O_2 + H^+ + 2\ H_2 + 2\ CO_2$	4	-296.18	-296.78	-291.65	-292.32	0.76	0.78	0.77	0.78
Acetate to propionate	$2\ C_2H_3O_2 + 2CO_2 + 6\ H_2 \leftrightarrow 2\ CH_3H_5O_2 + 4\ H_2O$	0	-70.64	-69.75	-75.38	-72.63	0	0	0	0
Acetate to butyrate	$2\ C_2H_3O_2 + H^+ + 2\ H_2 \leftrightarrow C_4H_7O_2 + 2\ H_2O$	0	0.64	0.48	-0.40	1.25	0	0	0	0
Propionate to butyrate	$2\ C_3H_5O_2 + H^+ + 2\ H_2O \leftrightarrow C_4H_7O_2 + 2\ CO_2 + 4\ H_2$	0	71.28	70.24	74.98	73.88	0	0	0	0
Methanogenesis	CO2 + 4 H2 ↔ CH4 + 2 H2O	1	-112.38	-110.40	-115.31	-112.95	0.50	0.49	0.49	0.50
Acetogenesis	2 CO2 + 4 H2 ↔ C2H3O2 + H+ + 2 H2O	0.2	-72.11	-69.48	-73.07	-71.26	0.16	0.15	0.15	0.16
ADP to ATP	$ADP + P_i + H^+ \leftrightarrow ATP + H_2O$	1	56.57	56.69	56.45	56.87	--	--	--	--

[1]Data are calculated from (Ungerfeld et al., 2003) Table 2.
[2]Initial concentrations of acetoacetate (mM) from (Ungerfeld et al., 2003).

Table 7.9. Free energy (kJ/mol substrate) and efficiency (kJ/kJ) of rumen fermentation reactions when controlling for [glucose] with 6 mM acetoacetate[12].

			ΔG_{rxn}				$-\Delta G_{ATP}/\Delta G_{rxn}$			
Reaction	**Formula**	**No. ATP**	**[GLC][3] = 0.03**	**[GLC] = 0.3**	**[GLC] = 3.0**	**[GLC] = 30**	**[GLC] = 0.03**	**[GLC] = 0.3**	**[GLC] = 3.0**	**[GLC] = 30**
Glucose to acetate	$C_6H_{12}O_6$+ 2 H_2O <--> 2 $C_2H_3O_2$ + 2 H^+ + 2CO_2+ 4 H_2	4	-291.31	-297.26	-303.21	-309.17	0.78	0.76	0.75	0.73
Glucose to propionate	$C_6H_{12}O_6$ + 2 H_2 <--> 2 $C_3H_5O_2$ + 2 H^+ +2 H_2O	4	-361.06	-367.01	-372.97	-378.92	0.63	0.62	0.61	0.60
Glucose to butyrate	$C_6H_{12}O_6$ <--> $C_4H_7O_2$ + H^+ +2 H_2 + 2 CO_2	4	-290.82	-296.78	-302.73	-308.68	0.78	0.77	0.75	0.74
Acetate to propionate	2 $C_2H_3O_2$ + 2CO_2+ 6 H_2 <--> 2 $CH_3H_5O_2$ + 4 H_2O	0	-69.75	-69.75	-69.75	-69.75	0	0	0	0
Acetate to butyrate	2 $C_2H_3O_2$ + H^+ + 2 H_2 <--> $C_4H_7O_2$ + 2 H_2O	0	0.48	0.48	0.48	0.48	0	0	0	0
Propionate to butyrate	2 $C_3H_5O_2$ + H^+ + 2 H_2O <--> $C_4H_7O_2$ +2 CO_2 + 4 H_2	0	70.24	70.24	70.24	70.24	0	0	0	0
Methanogenesis	CO2 + 4 H2 ↔ CH4 + 2 H2O	1	-110.40	-110.40	-110.40	-110.40	0.51	0.51	0.51	0.51
Acetogenesis	2 CO_2 + 4 H_2 <--> $C_2H_3O_2$ + H^+ + 2 H_2O	0.2	-69.48	-69.48	-69.48	-69.48	0.16	0.16	0.16	0.16
ADP to ATP	ADP+ P_i + H^+ <--> ATP +H_2O	1	56.69	56.69	56.69	56.69	--	--	--	--

[1]Data are calculated from (Ungerfeld et al., 2003) Table 2.
[2]Initial concentrations of acetoacetate (mM) from (Ungerfeld et al., 2003).
[3] Model was run with the following [glucose] = 0.03, 0.3, 3.0, and 30 mmol/l

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BIOGRAPHICAL SKETCH

Latisha Marquita Judd was born on December 20, 1984 in Raleigh, NC. Latisha was raised in Holly Springs, NC with her older brother Larry and mother Linda Judd. Latisha was the first in her family to attend and graduate from a 4-year institution, North Carolina Central University in Durham, NC. During her time at NCCU, Latisha was active in several organizations including the, Honor Society, NCCU softball team, as well as Zeta Phi Beta Sorority Incorporated. It was during her time at NCCU in the Department of Biology that Latisha was introduced to research and began to prepare for attaining an advanced degree.

Latisha attended Virginia State University, Petersburg, VA to pursue her Masters degree in biology. It was at Virginia State that Latisha was introduced to small ruminant research where she worked with Spanish, Kiko, Boer, and Myotonic goats. Her thesis work focused on gene expression and resistance to parasites in goats. During her tenure here, Latisha's passion for teaching and education was stirred. Latisha served as a teaching assistant to the biology department for 4 semesters and took the time to tutor undergraduate students in addition to her coursework and research. Latisha's scholarship was recognized, and she was inducted into the Golden Key International Honor Society as well as Beta Beta Beta (TriBeta) National Biological Society. As is key in research, Latisha presented her thesis work at the Association of Research Directors 1890, Incorporated Meeting in Jacksonville, FL. It was at this conference that she was recruited to the University of Maryland to join the Department of Animal and Avian Sciences to further her studies as a Doctor of Philosophy student.

In August 2013, Latisha began her matriculation in pursuit of her Doctor of Philosophy degree. She had the privilege of joining the lab of Dr. Richard (Rick) Kohn in April 2014. Latisha's passion for research and teaching continued to blossom under the direction of Dr. Kohn. Her dissertation focuses on understanding rumen fermentation in vitro to shift fermentation towards more favorable products and reduce methane emissions in ruminants, specifically dairy cows. At the University of Maryland, Latisha has joined several organizations such as the Animal Sciences Graduate Student Association where she served as president during the 2015 – 2016 academic year. She was the department representative for Graduate Student Government, and served as the co-advisor to Minorities in Agriculture, Natural Resources, and Related Sciences (MANRRS) from 2016 – 2018. Latisha has mentored students as the co-advisor for this club in professional development and research presentation skills. Latisha has served on the Teaching and Learning Transformation Center's Advisory Board. In addition to her dissertation, Latisha is completing the University of Maryland's University Teaching and Learning Program's scholar level. She has implemented an Institutional Review Board approved research study in a 200-level animal physiology course. Upon completion of the program Latisha will receive a notation on her transcript indicating she has professional training in teaching. Latisha has received numerous awards and fellowships throughout her matriculation for research in addition to travel grants to present her research at national meetings. Latisha was the recipient of the following awards in 2018: Outstanding Ph. D. Student of the Year (Department of Animal and Avian Sciences), AGNR Alumni Outstanding Graduate Student in the Animal and Avian Sciences Department, AGNR Alumni Outstanding Graduate Student, College of Agriculture and Natural Resources, Outstanding Graduate

Student Distinguished Service Finalist, Graduate Student Award, and President's Commission on Ethnic Minority Issues. Latisha was invited to join Gamma Sigma Delta Agricultural Honor Society in 2015. In addition to this scholarly recognition, Latisha belongs to the following professional organizations: American Association for Agricultural Education (AAAE), American Society of Animal Sciences (ASAS), and the American Dairy Science Association (ADSA). Upon completion of her degree, Latisha plans to seek employment in a tenure-track faculty position focusing on ruminant nutrition, research, teaching, and extension.