ABSTRACT

Title of Dissertation: MECHANISMS CONTROLLING VOLATILE FATTY ACID AND FERMENTATION GAS PRODUCTION IN THE RUMEN

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Atmospheric methane accounts for less than 16% of global anthropogenic greenhouse gas emission, while methane from ruminant species accounts for a small fraction of the total atmospheric methane. Enteric methane production also results in major dietary energy loss and reduces feed efficiency or overall farm productivity. Studies investigated numerous feed additives with variable findings on anti-methanogenic properties of these compounds or feeding strategies. However, the findings raised questions regarding observed shifts in VFA profiles. Higher inclusion levels of concentrate in the diet of ruminants decrease methane production and shift VFA profiles. Many studies have suggested that inhibiting methanogenesis avails reducing equivalent for the propionate-producing pathway that results in shifts in VFA profiles. Microbial Kinetics and thermodynamics are physiochemical principles that may be used to study how concentrate inclusion change the substrate availability and lead to shifts in fermentation profile. Substrate availability also support the growth of rumen microbial population, while accumulation of products or reactants for major

fermentation pathways dictate the profile of VFA. Studying how these competing concentrations change the production rates would help researchers understand how VFA profiles are shifting during methane inhibition and help to identify a more targeted approach for inhibiting methane. The research objectives were; to develop an in vitro method to understand the basal kinetic parameters of H_2 metabolism in the rumen, to evaluate the effects of increasing forage-to-concentrate ratio on performance and change in VFA and fermentation gas in vivo, and to test the effect of various perturbations (fermentation metabolites e.g. sodium acetate, sodium lactate etc.) on the fermentation profile of rumen fluid adjusted to different forage-to-concentrate ration. The results indicate that rumen fluid from cows on a high-concentrate diet have a greater capacity to make propionate compared to the high forages diet. The higher propionate production limits the availability of H_2 which is necessary for the synthesis of CH_4. The findings also suggest that methanogenesis is limited by substrate concentration. Finally, our studies indicate that feeding strategies targeting enzymatic activity favoring propionate production may be more beneficial than targeting methanogens in a high forage diet.

MECHANISMS CONTROLLING VOLATILE FATTY ACID AND

FERMENTATION GAS PRODUCTION IN THE RUMEN

by

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Dedication

Dedicated with much love to Gregory, Veronica, Jerome, Jessica, Judeen and Joan

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LITURATURE REVIEW

Anaerobic fermentation and the rumen

The ruminant digestive system has evolved over the centuries to be a highly efficient fermentation chamber capable of metabolizing high fiber diets with the aid of a diverse microbial population. This has resulted in ruminants being able to utilize feed otherwise considered to have low nutrition value for monogastric organisms. The feed mainly consists of complex and simple sugars, proteins, lipids and minerals which are digested to simpler constituents by bacteria, fungi and protozoa present in the rumen. These smaller components are then fermented to volatile fatty acids (VFA) which serve as the most important end product of rumen fermentation and serve as the main energetic precursor for many metabolic processes in the cow (Storm, Kristensen, et al., 2012). There are numerous factors affecting the profile of fermentation gases and the ruminal concentrations of acetate, propionate and butyrate, chiefly, the offered diet greatly influences the profile of VFA and fermentation gases (Bharanidharan, Arokiyaraj, et al., 2018).

There are many different pathways for VFA synthesis, polysaccharides are mainly metabolized to monosaccharides and then pyruvate which is quickly converted to the acetate, propionate and butyrate. Pyruvate's conversion to acetate and butyrate is heavily dependent on acetyl CoA as an intermediate, while propionate is produced mainly via the succinate pathway and occasionally through the acrylate/lactic acid pathway (France and Dijkstra, 2005; Ungerfeld and Kohn, 2006). In addition to the VFA production, ruminal fermentation also leads to the production of fermentation gases, namely CO_2, CH_4, and ammonia, with smaller quantities of formate, ethanol, lactate and succinate (Krause and Oetzel, 2005; Haque, 2018).

Numerous studies have shown that altering the diet to supply higher concentrates reduces ruminal pH and increases VFA production, possibly from the increased availability of fermentable carbohydrates (Kumar & Kumar, 2016; Wang, Zhang, et al., 2020). Similarly, studies have shown that replacing forages with concentrates or high carbohydrate content reduces ruminal CH_4 concentrations (Martínez, Ranilla, et al., 2010; Knapp, Laur, et al., 2014).

Throughout the production life cycle of domesticated ruminants, the diet composition may shift between high and low concentrate-to-forage diets. As a typical management practice the diet of gestating dairy cows typically shifts from a low energy, high fiber diet to a high energy, high concentrate diet during the last 3 weeks of the non-lactating period (Roche et al., 2013). These shifts in diet promotes nutrition-induced changes in the rumen microflora necessary to alleviate the high post-partum demand of the mammary glands for glucose and energy (Loor, Elolimy, et al., 2016). Studies have shown that the changes in rumen microflora following the shifts to high energy diets increases the absorptive capacity and production of VFA, particularly acetate and propionate (Loor, 2016; Kim, Mamuad, et al., 2018).

Atmospheric Greenhouse Gas

Global climate change is a phenomenon explained by increased ocean and land temperatures over a long period of time. There have been numerous independent datasets showing the three-decade period between 1983 to 2012 was likely the warmest

30-year period for the northern hemisphere in the past 1400 years (IPCC, 2014). The constant increases in emission from both natural and anthropogenic sources of atmospheric greenhouse gas (GHG) has been identified as the driving force of climate change since the mid 20th century (Trenberth et al., 2007). Global GHG in the atmosphere primarily consists of carbon dioxide (CO_2), nitrous oxide (N_2O) and methane (CH_4), with global atmospheric percentages of 74.3, 6.2 and 17.3 respectively (Climatewatch 2019). The Intergovernmental Panel on Climate Change (IPCC's) last climate change review reported that CO_2, CH_4 and N_2O have shown significant increases of 40%, 150% and 20% respectively since 1750 (IPCC, 2014). These increases in GHG emission typically follow trends in population growth and increased industrialization and will continue if global emissions remain unaddressed and based on current predictions in population growth (EPA, 2020; Moss et.al., 2000)

Each GHG has an estimated warming potential that is used to quantify the radiative forcing (net change in energy flux) of a unit mass of gas relative to CO_2, therefore, the greater the warming potential the more the gas warms the earth's surface compared to CO_2 (IPCC, 2014). Additionally, CO_2 constitutes a larger portion of the global atmosphere and has the longest lifetime of all GHG, mostly remaining in the atmosphere for several thousands of years being recycled by the global carbon cycle (EPA, 2017). On the other hand, CH_4 has a shorter lifetime and remains in the atmosphere for 8-12 years before it is oxidized to CO_2 and water (Anenberg et al., 2012; EPA, 2017). This theory is confirmed in IPCC reports (2014) comparing the decadal rate of change the IPCC reported (2014) CO_2 concentrations having the fastest rate of increases between 2002 to 2011, while CH_4 concentrations remained stable between the early 1990's to 2007. However, while CH_4 concentrations are significantly lower in the atmosphere and has a shorter lifetime than CO_2, it has a warming potential that is at least 20 times that of CO_2 signifying the importance of reducing atmospheric methane percentages (GMI, 2010; Ramin & Huhtanen, 2012; Elanthamil & Bandeswaran, 2017).

One of the greatest issues with mitigating global GHG emissions is majority of the gases are produced as a byproduct of normal human activities including, but not limited to, transportation, generating electrical and heat energy, waste management, agriculture and industrial processes. Human activities associated with these societal enterprises contribute to the global GHG emission. Energy-related emissions accounts for 70% of the total GHG from combustion of natural gas, coal and oil with contributions primarily from electricity (25%), transportation (14%) and industrial (22%) sectors. Land use/ forestry and the agriculture sector accounted for 13% and 14% of the total GHG emissions. Similarly, the U.S. EPA (2018) reported comparable GHG emission trends for Transportation (28%), energy (27%), industrial (22%) and the agricultural (10%) sectors (Baumert et al., 2005; EPA, 2018). Therefore, while agricultural practices contribute a great deal to global GHG emissions, modification of many agricultural practices could result in reduction of GHG emissions and improve the removal of GHG from the atmosphere through carbon sequestration (Sands, 2022).

Enteric Methane

Methane emission from the agricultural sector accounted for 36.4% of the U.S total methane in 2020 and was ranked second to only the energy sector that accounted for 39.1% of the total U.S. methane emission (EPA, 2022). Majority of the global methane production are from anthropogenic sources including fossil fuel combustion, rice cultivation, manure management, coal mining and enteric methane production.

Enteric Methane is produced as a by-product of the fermentation of complex carbohydrates to VFA in the rumen and products of methanogenesis are expelled to the atmosphere as eructed fermentation gas. The complex microbial population of the rumen works together in methanogenesis, some by actual conversion of metabolites to methane, others synthesize precursors for various methanogenic pathways and some microbes play a key role in providing the ideal conditions for the growth and survival of methanogenic archaea (Morgavi, et al., 2010). However microbial archaea are mostly responsible for methane production (Cardoso-Gutierrez, Aranda-Aguirre, et al., 2021).

One of the main end-product of ruminal fermentation is volatile fatty acids which mostly comprises of acetate, propionate, and butyrate along with other longer chain fatty acids (Jan Dijkstra, Forbes, et al., 2005). VFA production in the rumen are merely products for ruminal microbes' effort to gain energy however these byproducts are extremely beneficial to the ruminants themselves as VFA serve as the primary energy source from fermentation. Enteric methane produced from the digestion process constitutes a small but metabolically expensive product synthesized to keep rumen hydrogen concentrations low (Van Lingen, Plugge, et al., 2016; Zhang, Wang, et al., 2020). Work conducted by Moss et al. (2000) demonstrated that oxidation of the reduced form of NAD^+ in the rumen may be an important step to ensure the appropriate environment for rumen fermentation. This process is partly supported by methane synthesis that utilizes free hydrogen in the system to drive both these reactions.

Anti-methanogenic Strategies

Recent intertest in understanding the impacts of enteric methane production on global greenhouse gas emissions has resulted in a variety of studies aimed at investigating numerous strategies to mitigate enteric methane emissions while improving performance in ruminants (Amat, McKinnon, et al., 2014; Patra & Yu, 2014; Yang, Rooke, et al., 2016; Doyle, Mbandlwa, et al., 2019; Wang, 2020; Pereira, De Lurdes Nunes, et al., 2022). A few trends in selected mitigation strategies will be highlighted in the upcoming sections.

Fat Supplementation

In addition to its importance in ruminal methanogenesis, metabolic hydrogen is also used as a mean to hydrogenate fatty acids in the rumen. Numerous experiments have had success with the inclusion of unsaturated fatty acid and decreased rumen methane production (Drehmel, Brown-Brandl, et al., 2018; Vargas, Andrés, et al., 2020). Before the use of carbohydrates as a major energy source dominated the ruminant diet formulation, fat sources were used and primary energy sources in the ruminant diets. Previous studies investigated the used of fat source in the form of sunflower oil as a possible anti-methanogenic, the findings showed that sunflower oil supplemented at 50g/kg of the DM of dairy cow diets was successful in reducing methane emission on a g/day as well ag/Kg or DM (Bayat, Ventto, et al., 2017). More recently, Vargas et al. (2020) tested the effects of different sources of fat (olive oil, sunflower oil and linseed oil) and all fat sources produced less methane on a mL/day and when expressed as mL/100mL of total gas. However, some research findings have suggested that dietary fats may be toxic to cellulolytic bacteria and have shown signs of impaired digestibility of carbohydrates. The suggested mode of action for methane inhibition under high fat conditions included enhanced propionate, direct inhibition of methanogens or the hydrogenation of the unsaturated fats in the rumen.

Ionophore Supplementations

Ionophores are a group of antimicrobial compounds that have been frequently used in ruminant production systems; they include compounds such as monensin, salinomycin, laidlomycin and lasalocid. While the sustained effects of ionophores as an anti-methanogenic compound is short-lived, additional benefits of dietary supplementation such as improved feed efficiency readily qualify ionophores for adoption as an economically viable and environmentally friendly feed supplement. Recent studies investigating the effects of methane sodium salt on the methane production of forage versus vinasse (highly digestible diet similar to high concentrate composition wise), showed significant reduction in methane concentration and production(Xue, Chen, et al., 2021). Additional studies reported up to 10% decrease in methane production (g/day) when high dose of monensin was added to the diets of dairy and beef cows(McGinn, Beauchemin, et al., 2004; Islam & Lee, 2019). Common ionophores such as monensin are also classified as antibiotics and conversations regarding antibiotic use and resistance may affects acceptability in the animal industry, solely driven by consumer preferences (Rosemond, 2021).

Diet composition

Dietary influences on mitigation of enteric methane have been widely studied and the findings have suggested that diets with higher inclusion of easily fermentable substrates favor lower methane production compared to diets with higher fiber. As previously discussed, the availability of hydrogen in the rumen is positively associated with methane production and there are numerous feeding strategies favoring the production of propionate and butyrate over acetate production which will perturb methanogenesis in the rumen. Diets offered are the ultimate source of energy in the rumen environment, thus varying diet that offers different nutrients to the microbes and will ultimately dictate the VFA and gas profile (Knapp, 2014). Studies have also shown that methanogenesis decreases in the presence of high protein however increasing dietary fiber has been consistent in promoting enteric methane production (Johnson & Johnson, 1995).

Increased propionate production is also associated with higher inclusion of concentrates in the diet and propionate serves as a precursor for the synthesis of glucose and lactose. Redirecting metabolic hydrogen to these synthetic pathways improves the metabolic efficiencies and reduces energy loss to enteric methane eructation (Newbold, López, et al., 2005). Ideally, the most suitable forage to concentrate ratio depends on the physiological status, age and production system of the ruminants, hence these factors could also play a role in limiting the implementation of reduced forage inclusion as a methane mitigation strategy. A meta-analysis of 24 studies testing the effect of increasing concentrate inclusion in diets of different ruminant species decreased methane yield by 26%, 14% and 6 % in beef cattle, dairy cattle and sheep respectively (J. Dijkstra, Van Gastelen, et al., 2020)

Decreasing forage to concentrate ratio is accepted as a well-known strategy for mitigating enteric methane production. However, nutritional limitations such as acidosis, reduced milk fat, and laminitis hinders widespread use. Additionally studies have suggested that higher than normal propionate concentrations in the rumen could also have negative effects (Olijhoek, Hellwing, et al., 2016; Ungerfeld, 2020; Fouts, Honan, et al., 2022)

Influence of principles of thermodynamics and kinetics on rumen fermentation

All chemical reactions are controlled by kinetics and thermodynamics and the metabolic reactions leading to the production of VFA and gases in the rumen are no exemption to this rule (Chang, 1981; Kohn and Boston, 2000). Kinetics refers to the key role of enzyme activity and substrate concentration on specific rates of reactions. These rates of reaction dictate the ruminal profile of specific products within the rumen; however, the enzymes, reactant and product concentrations play an equally important role. Thermodynamics on the other hand deals with how stable a reaction and the propensity of that reaction to proceed in the forward direction towards the product or on a net basis towards the reactants. A metabolic reaction going from reactants to product is considered thermodynamically favorable if the free energy going from the product to the reactant decreases or if the Gibbs free energy (ΔG) becomes more negative. Jenssen (2010) explained, using ΔG, how increases in the concentration can result in a shift of VFA production away from acetate and to propionate synthesis by way of the electron accepting -pathway that is thermodynamically less favorable (Van Lingen, 2016).

Kinetic control is a key factor in the regulation of metabolic pathways and scientists have focused on using elements including substrate availability, enzymatic activity or microbial growth to manipulate system kinetics. Altering or limiting the function of any of these elements can result in delay or prevent the system from approaching thermodynamic equilibrium. In a biological system where all active enzyme sites have formed an enzyme-substrate complex, the system is said to be fully saturated or at its maximum velocity (V_{max}). Increasing the concentration of available substrate will not affect the velocity, this can only be achieved by increasing the enzyme concentration. A second kinetic parameter, the K_m, denotes the substrate concentration at which the rate or velocity is half of the maximum velocity or V_{max}. Monod (1949) demonstrated that these kinetic parameters can be used to quantify changes in the microbial activity of a system. Therefore, studies investigating what changes occur in a living system as a result of changes in the microbial activity must consider these kinetic parameters.

Jin and Bethke (2007) used the thermodynamic potential factor to quantify the thermodynamic control on rumen fermentation rates. The typical rate laws were used and they accounted for the differences between the available and conserved energy through fermentation (Van Lingen, 2016). The spontaneity of a reaction is determined by the calculated ΔG and studies have calculated ΔG of the products in the rumen under normal metabolite concentrations, showing ΔG's at or near zero (Kohn and Boston, 2000; Ungerfeld and Kohn, 2006). This highlighted that thermodynamic rather than kinetics is the driving force behind the profile of fermentation products. Hence, the influence of reactants product concentration is greater than that of the enzymatic activity.

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Increasing headspace hydrogen concentration increased the activity of hydrogenotrophic methanogens in rumen fluid and shifted fermentation profiles *in vitro*

ABSTRACT

The complexity of the microbial population in the rumen and its response to methane inhibitions has been studied extensively, both *in vitro* and vivo. However, there are numerous questions regarding the mode of action of methane inhibition in the rumen and identifying the most sustainable anti-methanogenic factors while identifying ways to capture the energy expended in methanogenesis. The current experiment focused on developing the most suitable conditions to study the kinetic and thermodynamic parameters of methanogenesis using increasing headspace as substrate. Secondly the developed method was used to test the effect of increasing headspace on kinetic and thermodynamic parameters of the rumen. Experiment - I tested the effect2 of headspace (35 or 50%), incubations time (1, 3, 6, and 12 hours) and rumen fluid concentration (0, 1, 5, 10, 50 and 100 %). Increasing rumen fluid concentrations expectedly increased ($P < 0.05$) all VFA concentrations, fermentation gas productions and the rate of VFA formation, however, A:P ratio and CH_4, and CO_2 rate of reaction was unaffected ($P > 0.05$). Additionally, all substrate was utilized within 3- hours of incubation ($P < 0.05$) regardless of headspace $_{added}$ (35 or 50%). Experiment - II tested the effect of hay addition (Hay or No Hay), headspace (35 or 50%) and incubation time (4, 8, 16 and 24 hours). Propionate, Butyrate, CH_4, and CO_2 concentrations as well as A:P and rate of reaction for CH_4 and increased ($P < 0.05$) between 0 and 4hrs of incubation but remained constant after 4-hours of incubation. However, total VFA, total gas production and the rates of reaction for all VFA were unaffected ($P > 0.05$) throughout

the experiment. Hay addition increased all fermentation parameters however the headspace had no effect ($P > 0.05$). The third experiment tested the effect of increasing levels of hydrogen (5, 15, 30 and 75) while incubating for 4 hours with Hay addition. There was a linear increase ($P < 0.05$) CH_4 production and disappearance, additionally CH_4 rate of formation had a tendency ($P = 0.07$) to increase with increasing headspace (approx. 0, 0.06, 0.58 and 1.57 mmol hour^{-1} for 5, 15, 30 and 75% respectively), while acetogenesis reached maximum rate of formation (Michaelis-Menten V_{max}) of 8.69 mmol hour^{-1} at 0.90 mmol of . Lineweaver- Burk transformation of data yielded V_{max} of 11.61 and 0.65 mmol hour^{-1} for acetate and CH_4 respectively. Additionally, ΔG values ranging from -105.32 to -115.60 kJ for methanogenesis and -71.16 to 81.03 kJ for reductive acetogenesis demonstrated the thermodynamic feasibility of both reactions under conditions of Experiment - III. The results from these experiments suggest that increasing headspace concentrations up to 35%, incubations time ≤ 4 hours and the presence of hay may be considered the most ideal conditions for studying rumen kinetics *in vitro*. Additionally, rumen methanogenesis appears to be limited by enzyme concentration (methanogen growth) and reductive acetogenesis appears to be feasible only with H_2 above certain concentrations.

INTRODUCTION

Increased interest in anthropogenic sources of global greenhouse gas emission (GHG) has shifted consumer choices and heavily influenced research interest and recent changes in policy (Thoma, Popp, et al., 2013; Pereira, De Lurdes Nunes, et al., 2022). These changes have resulted in major adjustments in how agricultural goods are produced with particular interest being directed to large scale livestock production systems and enteric methane (CH_4) emissions. In addition to the environmental impacts, enteric methane emission accounts for 3-10% of gross dietary energy loss and negatively impacts feed efficiency and overall farm productivity (Ramin & Huhtanen, 2012; Lan & Yang, 2019). A variety of anti-methanogenic compounds have been tested for their effectiveness in reducing enteric methane; these include the ionophores monensin and tannins (Witzig et al., 2018); use of electron acceptors such as nitrates (Vyas et al., 2018) and fumarate (López et al., 1999; García-Martínez et al., 2005); inclusion of various organic oils (Bayat et al., 2017; Judy et al., 2019) and increasing the levels or easily fermentable starch or grains (Hook et al., 2010; Aguerre et al., 2011; Bharanidharan et al., 2018). The results from these studies varied widely with respect to the mode of action for each anti-methanogenic compound. Additionally, results from these studies were inconsistent when comparing the relationship between methane inhibition and total volatile fatty acid (VFA) production, dry matter digestibility or A:P ratio. Although these studies were successful in identifying possible anti-methanogenic feed additives for the recovery of dietary energy expended in enteric methane production, the inconsistencies with the responses in other fermentation parameters has delayed widespread application off these techniques.

Anerobic digestion in the rumen starts with the digestion of structural carbohydrates, proteins and other organic matter that are degraded to monomers. The monomers are fermented to VFA that serve as important precursors in the synthesis of meat and milk. Other byproduct of the fermentation process includes metabolic CO_2 and , which are quicky converted to methane by the diverse population of methanogenic archaea in the rumen. The most common pathways typically used by these methanogenic bacteria are categorized by three major classes based on the electron doner being used; hydrogenotrophic, methylotrophic and acetolactic methanogens (Kim & Gadd, 2008; Pesta, 2015). However, the majority of the methanogens within the rumen are classified as hydrogenotrophic archaea and typically use H_2 as a reducing equivalent to reduce formate or CO_2 to CH_4 (Lieber et al., 2014; Patra et al., 2017). Recent studies have demonstrated the dependency of enteric methanogenesis on the availability of dissolved hydrogen and identified H_2 as the main substrate in methanogenesis (Olijhoek et al., 2016; Guyader et al., 2017; Greening et al., 2019; Emilio M. Ungerfeld, 2020). Though other methanogenic pathways using acetate, CO (carbon monoxide) or methylated substrates exist in the rumen, the apparent dependency of methanogenetic archaea on hydrogen suggests that H_2 gas may be used to study the kinetic control of methanogenesis and understanding the mechanisms behind methane inhibition in the rumen.

The rate of methane formation in the rumen is directly associated with the growth rate of the methanogenic species present. When all the nutrient requirements are met, the apparent correlation between the methanogenic growth rate and the available energy source or substrate for growth and the maximum methanogenic growth rate at infinite substrate availability is suggested to fit the principles of Monod and Michaelis Menten enzyme kinetic models (Conrad, 1999; Okpokwasili & Nweke, 2006; Janssen, 2010). Previous studies have attempted to fit the activity of hydrogenotrophic methanogens to Monod enzyme kinetic models however the results varied, and those experiments were conducted in continuous flow systems. Therefore, the current studies aimed to develop a repeatable *in vitro* batch culture method capable of 1) evaluating the basal effects of increasing headspace H_2 on kinetic parameter of fermentation gas and VFA *in vitro* and 2) identifying basal *in vitro* kinetic parameters on hydrogenotrophic methanogens.

MATERIALS AND METHODS

All experimental procedures were approved by the University of Maryland Institutional Animal Care and Use Committee (IACUC: Protocol number 1376980-2).

Rumen Fluid Collection

Rumen fluid was collected from rumen-fistulated non-lactating Holstein cows two hours before morning feed. Rumen fluid was processed according to the guidelines outlined in Goering and Van Soest (1970). Both solid and liquid fractions of the rumen content were sampled anaerobically in 50 mL centrifuge tubes and then capped for transportation to the lab. Total rumen fluid sample was homogenized by approximately 20 seconds of pulsing in a blender made anaerobically by flushing CO_2. Homogenized rumen fluid was strained through 4 layers of cheese cloth and a layer of glass wool, into a 1-L flask. Rumen fluid was stirred continuously using a magnetic stir bar and heated to 38°C while bubbling CO_2 to maintain rumen conditions until rumen fluid was distributed to culture tubes or bottles. Processed rumen fluid was distributed to Hungate tubes using a peristaltic pump or serological pipette depending on the desired volume.

Experiment - I

The first experiment (experiment - I) tested the effect of incubation time and microbial concentration on basal fermentation in the presence of two levels of headspace H_2 percentages. The treatments were arranged as a 2 × 5 × 6 factorial in a completely randomized design (CRD); the factors were 2 levels of headspace H_2 (35.5% H_2 or 50 % H_2), 5 different timepoints (0, 1, 3, 6 or 12 hours of incubation) and 6 different levels of rumen fluid (0, 1, 5, 10, 50 or 100%). All Hungate tubes (20 mL) were made anaerobic by CO_2 perfusion and filled with 10 mL of experimental media (pure *in vitro* media, pure rumen fluid or various mixtures of the two) using an autosampler before being stopped with a butyl stopper and capped. The varying levels of microbial population was achieved by mixing rumen fluid (RF) and buffered *in vitro* media (IV media) at ratios of 0:10, 0.1:9.9, 0.5:9.5, 1:9, 5:5 and 10:0 of rumen fluid: IV media. Glass syringes were used to add and mix H_2 (6 and 10 mL) to the headspace of tube resulting in 35.5 and 50 % H_2. Hungate tubes were incubated for 5 minutes while shaking at 39°C to facilitate microbial adaptation. After the microbial adaptation, a 27-gauge needle fitted to a 2-way Luer-Lock stopcock valve and a 20-ml air-tight Fortuna glass syringe was used to sample the 0hr headspace gas in each bottle and liquid (~1.5

ml) was collected for 0hr VFA analysis. Subsequent VFA and headspace gas was collected 3, 6 and 12 hours after initial sample collection and stored for later analysis.

Experiment - II

A third experiment tested the *in vitro* effects of the presence of hay on the fermentation profile in the presence of two levels of H_2. The experimental design was a 2 × 5 × 2 completely randomized design (CRD) factorial with 2 levels of headspace H_2 (35.5% H_2 or 50 % H_2), 5 different timepoints (1, 4, 8, 16 or 24 hours of incubation) and the addition of hay (0 or 0.10 g of timothy hay) as the factors. Hungate tubes (20 mL) were perfused with CO_2 for approximately 10 minutes and processed rumen fluid (10 mL) was added to each Hungate tube, any CO_2 gas displaced from the addition of rumen fluid was discarded. Glass syringes were used to add and mix H_2 (6 and 10 mL) as described for experiment I. Additional VFA and headspace gas was collected 8, 16 and 24 hours (8hr, 16hr and 24hr) after initial sample collection and stored for analysis.

Experiment - III

The third experiment tested the effect of increasing headspace H_2 concentration on the fermentation using appropriate rumen fluid concentration and incubation time. A completely randomized design (CRD) was used as the experimental design and 4 different levels of headspace H_2 (5, 15, 30 and 75 % H_2). Each Wheaton bottle received 50 mL of experimental media (25 mL of IV media and 25 mL of processed rumen fluid) and the headspace gas treatment was administered following the volumes outlined in Table 1.1.

Initial VFA and headspace gas samples were collected after 10 minutes of shaking at 39°C and stored for later analysis. Additional VFA and headspace gas were collected after 3 hours of incubation and stored for analysis. Individual rate of formation for each VFA and gas were calculated and fitted to the Monod/Michaelis-Menten kinetic model. For those reaction rates where reaction velocity increased with substrate concentration to a maximum, the Lineweaver-Burk plot was used to estimate the kinetic parameters. The inverse velocity (1/velocity) was regressed against the inverse reciprocal for substrate (hydrogen concentration). Maximum velocity (V_{max}) was calculated as 1/ y-intercept, while K_m was calculated as -1/x-intercept.

VFA Analysis

Stored rumen fluid samples were analyzed for VFA concentration using gas chromatography. The VFA samples were prepared using a modification of the method described by Erwin et al. (1961) where rumen fluid samples were thawed at room temperature and centrifuged at 12,000 × g for 30 minutes at 4°C. 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 1.8 m length x ¼ in. outer diameter x 4 mm inner diameter packed GC column (60/80 Carbopack-11766 support, model 1-21641, 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 29 mL/min. The initial column temperature was 110°C held for 1 min, then increased to 180 °C (ramp of 10°C/min) for 1 min, then increased to 200 °C (ramp of 20°C/min) for 3 min, and a post-run temperature of 110°C. The detector temperature was 250°C with a hydrogen and air flow of 30 mL/min and 200 mL/min, respectively. VFA rate of formation was reported as mmol Hour $^{-1}$ and calculated using the following formula:

$$\text{VFA Rate of Formation (ROF)} = \frac{\Delta \text{ VFA concentration (mmol)}}{\Delta \text{ Time (Hr)}}$$

Headspace Gas Collection

Headspace gas was collected by connecting a 3-way Luer-lock stopcock valve to an empty 100 mL Fortuna glass syringe. A 27-gauge needle was connected to the opposite end of the 3-way Luer-lock stopcock, and an additional labeled plastic syringe fitted with a 2-way stopcock was attached to the third/perpendicular end of the 3-way stopcock. The post incubation headspace gas volume was measured by closing the valve on the plastic syringe and allowing excess gas to pass into the 100 mL glass syringe. After the total gas volume was recorded approximately 10 mL of gas was subsampled by transferring from the glass syringe to the plastic syringe for later analysis (within 48 hours).

Headspace Gas Analysis

Gas concentrations were measured using gas chromatography (Hewlett-Packard model 6890) with a 15.0 ft length × 1/8 in. outer diameter × 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). Argon was used as a carrier gas with a constant pressure at 35.0 psi, flow of 6.5 mL/min. The column remained isothermal

150°C and held for 9.5 min. Detector was set to 250°C with a reference flow of 56.0 mL/min and makeup gas (Helium) set to 7.0 mL/min. Individual gas rate of formation was reported as mmol Hour $^{-1}$ and calculated using the following formula:

$$\text{Gas Rate of Formation (ROF)} = \frac{\Delta \text{ Gas concentration (mmol)}}{\Delta \text{ Time (Hr)}}$$

Statistical Analysis

Statistical analyses were conducted using JMP Pro 15 (JMP®, Version 15.2.0 SAS Institute Inc., Cary, NC, 1989-2007). The model statement for Experiment -I was: $Y_{ijk} = m + H_i + RF_j + T_k + (H \times RF)_{ij}\ \ (H \times T)_{ik} + e_{ijk}$ where H, RF and T represented the fixed effects of headspace hydrogen, rumen fluid concentration and incubation time respectively. The model for Experiment -II was $Y_{ijk} = m + H_i + G_j + T_K + (G \times T)_{jk} + e_{ijk}$ where H = headspace hydrogen, G = addition of hay and T = incubation time. Experiment -III was: $Y_{ij} = m + H_i + e_{ij}$ where Y_{ij} where the response variable H was the fixed effect of headspace hydrogen concentration. Statistical differences were.05 and a tendency toward statistical difference was declared at 0.05 < P £ 0.10.

RESULTS

Experiment - I

Increasing rumen fluid concentration and subsequently the microbial population resulted in an exponential increase in individual VFA concentrations and total VFA production *in vitro* (Table 2.2). Acetate production was 3.21, 4.01, 6.14, 8.63, 23.92 and 40.83 mmol at 0, 1, 5, 10, 50 and 100 % rumen fluid respectively, with a linear increase (P < 0.05) in acetate as rumen fluid concentration increased.

Propionate and butyrate concentrations were only different between 10, 50 and 100% rumen fluid concentration. However, similar trends were not observed for VFA rates of formation. Lower rumen fluid concentrations (0, 1, 5 and 10 %) had similar VFA rates of formation with acetate, propionate and butyrate producing 0.017, 0.05 and 0.04 mmol/hour respectively, however the rates of reactions increased as microbial concentrations increased to higher rumen fluid concentration. Additional differences ($P < 0.05$) were observed with ROF doubled between 50 and 100% rumen fluid for propionate and butyrate ROF (Table 2.2). Increasing rumen fluid concentration had no effect ($P > 0.05$) the H_2, CH_4 or CO_2 ROF. However, H_2 concentration decreased significantly ($P < 0.05$) as rumen fluid inclusion increased, with 3.97, 4.38, 3.89, 3.11, 2.32 and 1.66 ml/ 10 ml for 0, 1, 5, 10, 50 and 100% rumen fluid therefore suggesting increased H_2 utilization with increasing concentration on microbial population. Methane concentrations were only significantly higher ($P < 0.05$) for the higher inclusion of rumen fluid in the fermentation media (50 and 100). While CO_2 concentration and total gas volume were lowest with the highest inclusion on rumen fluid in the fermentation media.

All VFA concentrations, total VFA, CH_4, CO_2 and total gas production increased ($P < 0.05$) with increasing incubation time (Table 2.3). Hydrogen concentration decreased ($P < 0.05$) from 7.36 mL at 0 hrs to 3.11, 2.90, 1.13 and 1.49 after 1, 3, 6 and 12 hrs of incubation respectively. There was also a significant interaction ($P = 0.05$) between added hydrogen and incubation time. Headspace hydrogen for the 50% H_2 treatment remained approximately 4 mmol greater than the 37.5% H_2 treatment for 0, 1 and 3 hrs of incubation, however, there was no significant difference between treatment group headspace concentration (2 mmol/ 10 mL of rumen fluid) for 6 and 12 hrs (Figure 2.1). Rates of formation for all VFA and fermentation gas was significantly higher between 1 and 3 hours of incubation with a reduction in reaction rates as time progressed.

Experiment - II

The addition of timothy hay to Hungate tubes in experiment – II improved overall fermentation. The individual concentration of acetate, propionate and butyrate and total VFA (mmol) as well as the H_2, CH_4 and CO_2 volume (mL) were significantly higher for the fermentations tubes with hay added. Acetate, propionate and butyrate rate of formation was not affected ($P > 0.05$) by the presence of hay in the present *in vitro*. Additionally, the production of fermentation gases was significantly higher for both the concentration and rates of formation of H_2, CH_4 and CO_2 (mmol hour^{-1}). Under the current conditions the two different levels of treatment gas (35.5% or 50%) had no effect ($P > 0.05$) on either individual concentration or rates of formation for VFA and fermentation gas (Table 2.4).

There was a linear increase in the concentration of butyrate as incubation time increased. Similarly, propionate had a tendency ($P = 0.08$) of increasing from 5.36 mmol to 5.62, 6.12, 6.53 and 7.09 mmol for 0, 4, 8, 16 and 24 hours of incubation respectively. However, there was no effect ($P > 0.05$) of incubation time on the concentration of acetate or total VFA volume in Experiment – II therefore A:P ratio declined with increasing incubation time. All available substrate (H_2 gas) was depleted by 4 hours of incubation, highlighted by the negative standard curve estimates for H_2

volume at 4, 8. 16, and 24 hours of incubation (Table 2.5). Methane concentrations increased from 0, 2.13 to 2.66 mL 10 mL of rumen fluid^{-1} for 0, 4 and 8 hours of incubation but remained constant as incubation time increased to 16 and 24 hrs of incubation.

There was no significant (P < 0.05) effect of incubation time on the rate of formation for each VFA and CO_2 (Table 2.5). However, CH_4 formation and H_2 disappearance increased to the maximum rate of reaction (0.03 and 0.14 mmol Hour^{-1} respectively) between 4 and 8 hours of incubation followed by significantly lower rates of reaction between 16 and 24 hours of incubation.

Experiment - III

Increasing starting headspace H_2 concentrations was confirmed with initial H_2 volume for 5, 15,30 and 75 % increasing (P < 0.05) from 15.82, 26.23, 44.61 and 81.70 mL respectively. Increasing the H_2 in the headspace also resulted in increasing CH_4 volume, with headspace CH_4 increasing from 1.66, 2.18, 4.65 and 8.34 mL for 5, 15, 30 and 75 % addition of Hydrogen (Table 2.6). There was no significant difference (P > 0.05) observed for acetate, propionate, butyrate and total VFA concentrations. Similarly, there were no observed differences in A:P ratio, final Hydrogen or total gas production. Total gas production remained constant (P > 0.05) across treatment, producing 134.52, 138.07, 136.16 and 147.61 mL of gas with the addition of 5, 15, 30 or 75% headspace H_2 (Table 2.6). Similarly, methane production (Δ CH_4) and hydrogen disappearance (Δ CH_4) increased with increasing levels of headspace H_2. Methane production more than doubled in mmol from 0.01, 0.09, 0.22 and 0.41 for 5, 15, 30 and

75% hydrogen in the experiment. Additionally, methane production expressed as a fraction of hydrogen disappearance increased linearly (-4.67, 3.17, 12.29 and 18.90 mmol mmol^{-1}) with increasing levels of headspace hydrogen).

Acetate rate of formation increased from 3.82 mmol hour^{-1} at 0.30 mmol or 5 % H_2 to 8.58, 9.06 and 8.42 mmol hour^{-1} at for 0.90, 1.79 and 4.49 mmol of headspace H_2 respectively. Similarly, CH_4 rate of formation had a tendency (P = 0.08) to increase after 0.90 mmol of hydrogen but never reaches a point of saturation. Methane rate of formation ranged from -0.51, 0.07, 0.58, 1.57 mmol hour^{-1} for 0.30, 0.90, 1.79 and 4.49 mmol. Increasing headspace H_2 had no significant effect on the rates of formation for butyrate (P = 0.41) and propionate (P = 0.73), although propionate rate for formation increased numerically with increasing H_2 (1.22, 1.75, 2.05, 2.09 mmol h^{-1}).

Line-weaver Burk's plot was used to extrapolate the kinetic parameters for the influence of headspace H_2 on VFA formation (Figure 2.3) and methanogenesis (Figure 2.4). Acetate maximum rate of formation (V_{max}) based on the transformation was 11.61 mmol hour^{-1} with a k_m of 0.58 mmol of H_2 (Table 2.7). Propionate ROF ranged from 1.22 mmol hour^{-1} at 0.3 mmol of H_2 to 1.76, 2.05 and 2.08 mmol hour^{-1} for 0.90, 1.79 and 4.49 mmol of H_2 respectively. Linear transformation of propionate ROF yielded V_{max} and k_m values of 2.268 mmol hour^{-1} and 1.32 mmol of H_2 concentration (Table 2.7). Similarly, butyrate ROF achieved maximum enzyme saturation ($P > 0.05$) at H_2 concentrations below 0.30 mmol hour^{-1} with 0.18, 0.50, 0.72 and 0.53 mmol hour^{-1} of butyrate for 0.30, 0.90, 1.79 and 4.49 mmol of H_2. Lineweaver Burk transformation resulted in kinetic parameters (V_{max} of 1.004 mmol hour^{-1} and k_m of 1.315 mmol of H_2) that also support the Michaelis Menten plot in Figure 2.2.

The Lineweaver Burk linear transformations in Figure 1.4 plots the inverse velocity of methanogenesis against the inverse substrate concentration of four separate experiments where the asymptote or Vmax of methanogenesis was not achieved. Individual methanogenic Vmax and Km for various preliminary experiment are included in Table 2.7 with an average Vmax of 0.425 ± 0.172 mmol hour^{-1} and a k_m of 0.0198 ± 0.22 mmol of headspace H_2. The change in free energy for various fermentation reactions was calculated using calculated using the following equation:

$$\Delta G = \Delta G^{\circ} + RT \ln \{[Products] / [Reactants]\}$$

Increasing headspace H_2 had no influence (P = 0.18) on the ΔG for methanogenesis with estimates of -105.32, -111.62, -115.60 and -106.63 kJ calculated for 5, 15, 30 and 75 % headspace H_2 respectively. The ΔG for the use of headspace H_2 in reductive acetogenesis tended (P = 0.06) to be more negative for 15 and 30% headspace H_2, suggesting that the reaction was most feasible at 15 and 30% compared to other headspace H_2. Additionally, the conversion of acetate to propionate was most feasible (P = 0.05) for 13, 30 and 75 % headspace H_2 compared to rumen fluid incubated with 5% headspace H_2. The positive ΔG for conversion of propionate to acetate under the conditions of this experiment suggests that the reaction could feasibly proceed in the opposite direction.

DISCUSSION

Preliminary experiments were conducted testing the effects on increasing headspace H_2 concentration on basal rumen fermentation kinetics, particularly the rates of formation of acetate and methane, as well as to demonstrate the role of thermodynamic principles in dictating the profile of fermentation gases and VFA. These experiments suggested that most of the headspace H_2 disappeared within the first 2 hours of incubation and prevented accurate estimation of the final H_2 concentration and ultimately the rate of H_2 disappearance. The costs, tedious time consumption and infrastructural necessities associated with *in vivo* ruminant nutrition studies has resulted in widespread acceptance of *in vitro* nutrition experimental techniques. However, factors such as the rumen fluid to buffer ratio of the fermentation media is suggested to be one of the many limitations affecting reproducibility and consistency in fermentation studies. To effectively follow the disappearance of H_2 and rates of reaction for methanogenic and acetogenic pathways, it was suggested that a reduction in microbial density used in Experiment - I may facilitate more accurate assessment of the fermentation kinetics for gas and VFA. Increasing microbial density has been shown to increase the rate of fermentation, up to a maximum rate (V_{max}) where further increases in the microbial density was ineffective at increasing fermentation rate (Hidayat et al., 1993). In Experiment - I there were no differences in gas/VFA concentrations and rates of formation in the lower rumen fluid concentration (0, 1 and in some cases 5%). However, higher rumen fluid inclusion (10, 50 and 100 %) in the fermentation media increased VFA concentrations and rates as well as the concentrations for individual fermentation gas. Increased availability of microbes for the conversion of substrates to fermentation products resulted in the observed linear increase for higher inclusion of rumen fluid. These results support previous findings where higher rumen fluid:buffer ratios improved fermentations parameters in both *in vitro* digestion studies (Navarro-Villa et al., 2011; Khan & Chaudhry, 2021) as well as biogas digesters (Budiyono et al., 2014). The rates of acetate, propionate and butyrate formation were similar between lower rumen fluid:buffer concentration (0, 1 and in some cases 5%), but increased linearly for the higher rumen fluid inclusion. This was partly due to the increased microbial density or presence of rumen fermenters in the fermentation media. The starting headspace H_2 concentration (16.7 and 22.3 M) were above the reported normal range for functioning rumen H_2 levels of 0.0001 to 0.05 mM based on the diet being offered and the ruminant species (Janssen, 2010) and had no effect on the fermentation gas or VFA in Experiment - I. Additionally, Experiment -I demonstrated that the maximum rate (V_{max}) of VFA and fermentation gas production, under the present conditions, was achieved between 1 and 3 hours of incubation. The majority of the available H_2 substrate, approximately 60.66 % of the starting total, was metabolized between 1 and 3 hours of incubation. It appears that under the conditions outlined in Experiment- II, if the headspace H_2 decreased to approximately 2ml per 10 ml of rumen fluid or 0.0089 mM (see Figure 2.1: between 1-3 hours of incubation) then production of gases and VFA were significantly affected.

Headspace was offered as the only source of substrate in previous fermentation experiments, however, methanogenesis in response to increased H_2 was limited under these conditions. Various studies have demonstrated the connection between digestible fiber and CH_4 production in anerobic fermentation(Aguerre et al., 2011; Bell & Eckard, 2012; Kadzere, 2018) where increasing dietary fiber or forage increasing CH_4 production both *in vitro* and *in vivo*. The inclusion of hay in Experiment – II increased CH_4 concentration and CH_4 rate of formation by approximately 50%. Furthermore, fiber/hay inclusion improved overall fermentation, indicated by increased VFA concentrations and rates of reaction with hay addition versus no addition. Bayat et. al (2017) conducted studies testing the effect of dietary fiber on fermentation and reported higher CH_4 Day^{-1} and CH_4 OMI^{-1} associated with the inclusion of dietary fiber. Furthermore, greater production of acetate is associate with fermentation in the presence of forage or a fiber source with H_2 being a major byproduct that is quickly used by methanogens to oxidize available CO_2 to CH_4. Additionally, headspace H_2 concentration in Experiment - III was mostly depleted within the 1- 3 hours of incubation and impeded further production of VFA and gases beyond the 3-hour threshold. Suggesting that future fermentation experiment be terminated after 3-4 hours of incubation if an easily metabolizable substrate such as H_2 gas is used as substrate.

A final experiment was conducted (Tral – III), using the appropriate incubation time, rumen fluid concentration and headspace H_2 concentration based on previous studies. Wheaton bottles were used to increase the total volume of headspace gas available and the volume of H_2 added per mL of rumen fluid to ensure available H_2 was not metabolized in the 3-hour incubation period. Increasing headspace H_2 concentration in Experiment – III did not affect individual VFA concentrations, A:P ratio, total VFA concentration among other fermentation parameters. However, there was a significant increase in CH_4 (mL), ΔCH_4 (mmol) and ΔH_2 (mmol) with increasing starting headspace H_2 concentration. These finding support previous findings where dissolved hydrogen (dH_2) concentrations was positively correlated with growth of methanogens and methane production in rumen fluid (Martinez-Fernandez et al., 2016; Wang et al., 2017). Similar findings were observed with *in vitro* studies where increasing headspace H_2 increased methane concentrations and methanogenic microbial population (Qiao et al., 2015). Additional experiments demonstrated this relationship when feeding elemental Mg^+ increased dissolved H_2 and subsequently increased methane production in the rumen of high producing dairy cattle (Ma et al., 2019).

Acetogenesis in the final experiment (Figure 1.2-A) appears to fit a typical Michaelis-Menten kinetic model and achieved a maximum rate of formation (V_{max}) of approximately 9.69 mmol hour^{-1} at 0.9mmol headspace H_2. Transformation of the acetogenic rate of formation using Lineweaver Burk plot yielded higher values for V_{max} (11.61 mmol hour^{-1}) and a k_m (0.58 mmol of H_2) compared to the Michaelis-Menten plot estimates. Methane rate of formation followed a typical first order reactions with increasing methane reaction rates as headspace H_2 levels increased, without reaching an asymptote or achieving V_{max}. This indicates that methanogenic enzymatic activity was not limited in the rumen fluid for Experiment - III. Lineweaver-Burk transformations of methane ROF yielded a methanogenic maximum reaction rate (V_{max}) of 0.65 mmol hour^{-1}. Previous studies attempting to estimate values for the kinetic parameters have shown major inconsistencies (Strayer & Tiedje, 1978; Robinson & Tiedje, 1982; Janssen, 2010)

Further association between increased headspace H_2 addition and H_2 disappearance or CH_4 formation was thermodynamically supported in this study with more negative estimates for ΔG methanogenesis. These calculations suggest that as we increase H_2 in the headspace it becomes more thermodynamically feasible for disappearing hydrogen to be incorporated in methane than any other pathway outlined in Table 2.8. This feasibility for excess H_2 to utilized by methanogens versus reductive acetogens was also theoretically demonstrated theoretically by Ungerfeld and Kohn (2006) and in other experimental experiments (Heise et al., 1989; Liu & Whitman, 2008).

Previous meta-analysis estimated typical rumen H_2 concentrations, based on the solubility of hydrogen at 39°C, would range from 0.09 to 0.25 mmol (Hegarty & Gerdes, 1999). Therefore, the lowest substrate concentration in Experiment - III was above normal reported levels of headspace H_2 in the rumen and excess headspace H_2 conditions along with the calculated ΔG for reductive acetogenesis suggest that reductive acetogenesis may account for the increased acetogenesis between 0.3 and 0.9 mmol headspace H_2. Furthermore, studies have identified reductive acetogenesis as an important sink for excessive hydrogen and carbon dioxide in the gut fermentation (Godwin et al., 2014).

Incubation time and substrate concentration are both factors influencing the growth rate of microbes, thus the four-hour incubation was sufficient to facilitate exponential methanogenic growth and methane production. However, the rumen also hosts comparably less reductive acetogens and the incubation time in the present study may not have been sufficient to facilitate the growth of these growing acetogens. At approximately 0.9 mmol of headspace H_2 the pathway serving as a major hydrogen sink switches from reductive acetogenesis to methanogenesis. Similar results were observed in studies comparing the dominant pathways and organisms involved in hydrogenotrophy of cattle and other foregut-fermenting species, where thermodynamically favorable reductive acetogenesis was favored over methanogenesis (Godwin et al., 2014).

CONCLUSION

Increasing headspace H_2 up to 35% of the Total headspace, in the presence of hay for 1 to 4-hour incubation time appears be the most ideal *in vitro* conditions for studying the basal kinetic and thermodynamic parameters of rumen fermentation. Additionally, these conditions demonstrated that rumen acetogenesis follows a typical Michaelis-Menten kinetic model while methanogenesis follows a first order kinetic model. Thermodynamic estimates also confirmed that reductive acetogenesis and acetate to propionate are all feasible reactions dictating the profile of VFA *in vitro*.

Table 2.1: Headspace gas preparation

Headspace H_2 %	**Added H_2 [2] (mL)**	**Added CO_2 [2] (mL)**	**Final H_2 [*] (mL)**
5	7	Flushed[1]	6.06
15	22	Flushed[1]	12.06
30	54	Flushed[1]	25.30
75	Flushed[1]	41	70.06

[1] Gas used for perfusion (124 mL)
[2] An air-tight Fortuna glass syringe fitted with 2-way Luer stopcock valve was filled with the respective gas treatment. Added gas was mixed with the flushed gas by repeated but steady pumping of the syringe's plunger.
[*] Final volume of H_2 in each 124 mL Wheaton bottle

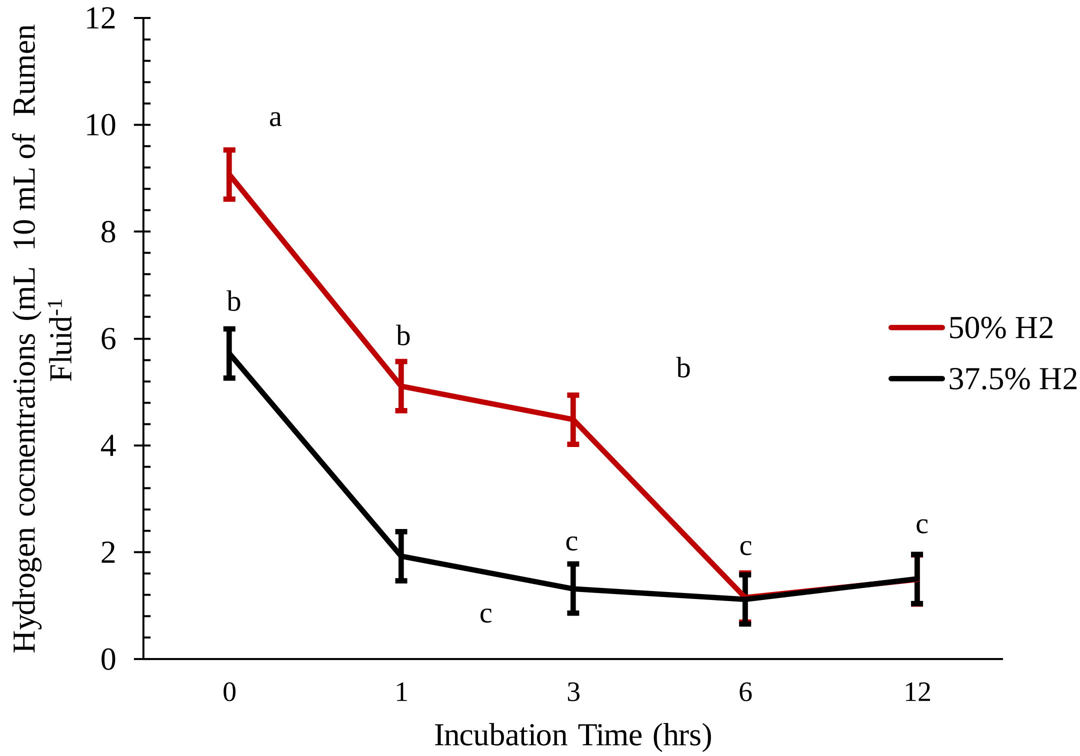
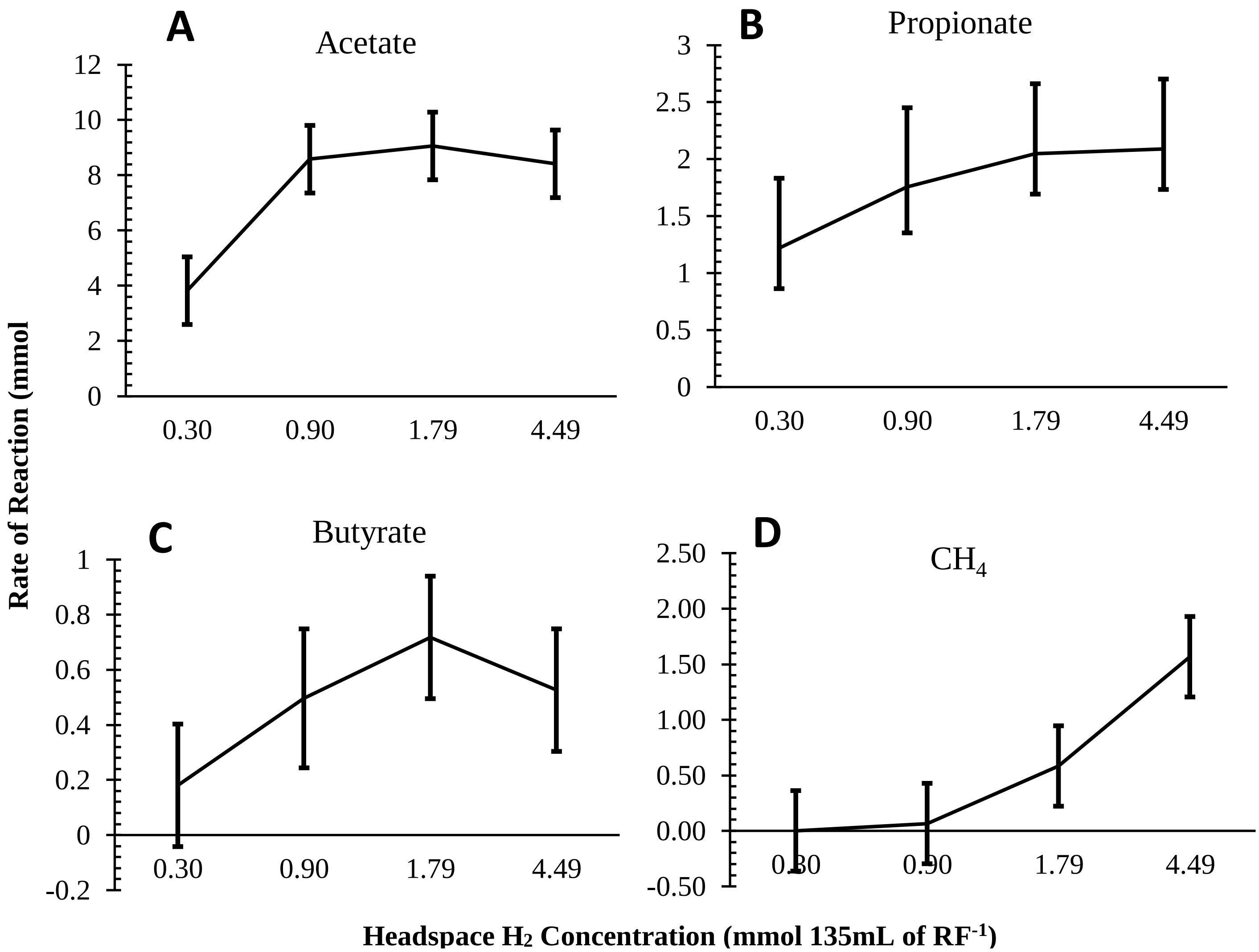


Figure 2.1: Effect of incubation time and added H_2 on headspace hydrogen concentration.



Individual graphs plot the velocity or rates of reaction in mmol hour $^{-1}$ (y-axis) against the increasing substrate concentration (H_2 %) for A) acetate, B) propionate, C) butyrate and D) methane -CH_4.
0.30, 0.90, 1.79 and 4.49 mmol of Headspace H_2 represents the hydrogen concentration at 5, 15, 30 and 75 %

Figure 2.2: The effect of increasing headspace H_2 concentration on rates of reaction for individual VFA and methane *in vitro*.

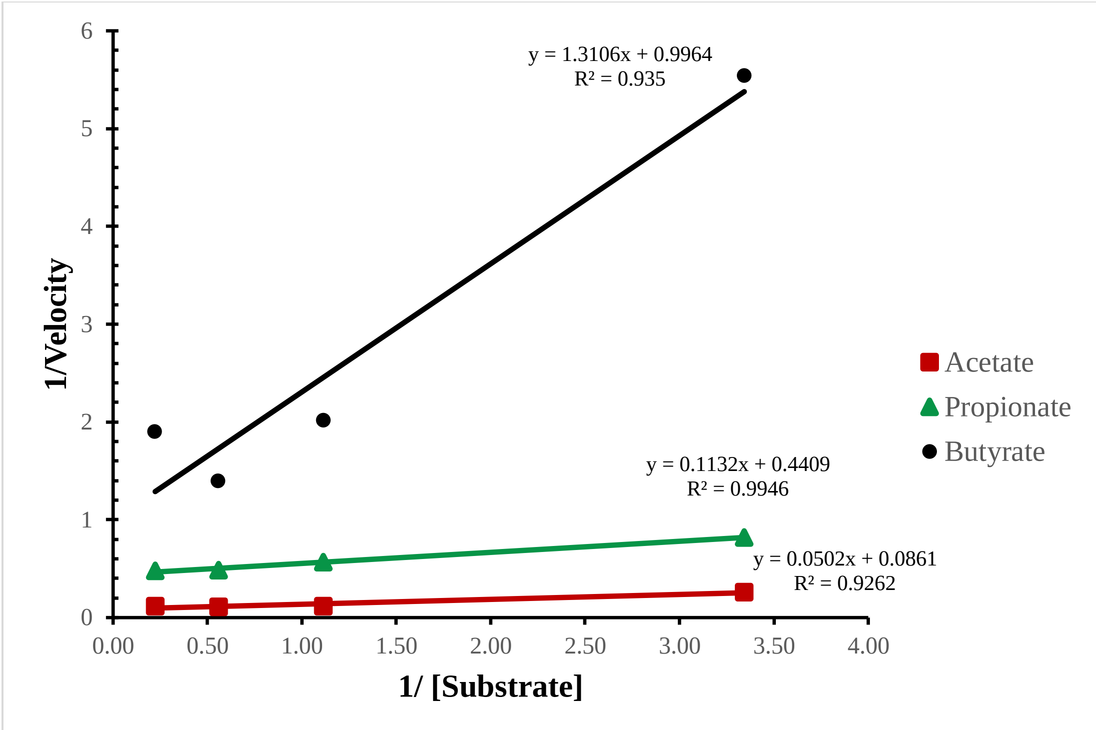


Figure 2.3: Lineweaver-Burk's plot of Experiment – III VFA rate of formation (velocity) as a function of substrate concentration.

Table 2.2: Effect of increasing rumen fluid concentration on *in vitro* volatile fatty acid and fermentation gas concentration in Experiment 1.

	Rumen Fluid Concentration[2]								**H_2 × RF**[1]
VFA	**0 %**	**1 %**	**5 %**	**10 %**	**50 %**	**100 %**	SEM	P-value	P-value
Acetate (mmol)	3.21[a]	4.01[a]	6.14[b]	8.63[c]	23.92[d]	40.83[e]	0.781	< 0.01	NS
Propionate (mmol)	1.00[a]	1.20[ab]	1.66[bc]	2.15[c]	5.78[d]	9.29[e]	0.334	< 0.01	NS
Butyrate (mmol)	0.37[a]	0.39[a]	0.47[a]	0.58[a]	1.28[b]	2.04[c]	0.118	< 0.01	NS
Total VFA	6.29[a]	7.31[a]	9.99[b]	13.07[c]	32.70[d]	53.88[e]	1.177	< 0.01	NS
A:P	5.00	4.90	4.76	4.86	4.60	4.67	0.200	NS	NS
Hydrogen (mL)	3.97[a]	4.38[a]	3.89[ab]	3.11[bc]	2.32[cd]	1.66[d]	0.456	< 0.01	0.05
Methane (mL)	0.00[a]	-2.8e-17[a]	0.03[a]	0.19[ab]	0.69[c]	0.47[bc]	0.136	< 0.01	NS
Carbon Dioxide (mL)	11.97[b]	15.25[a]	15.27[a]	14.86[a]	13.97[a]	13.19[ab]	1.011	0.0202	NS
Total Gas Volume	18.04[b]	19.63[a]	19.19[ab]	18.16[b]	16.98[c]	15.32[d]	0.502	< 0.01	NS
Rate of Formation (mmol Hour $^{-1}$)									
Acetate	0.05[b]	0.28[b]	0.05[b]	0.29[b]	1.00[a]	1.19[a]	0.267	< 0.01	NS
Propionate	0.03[c]	0.08[bc]	0.01[bc]	0.07[bc]	0.20[b]	0.49[a]	0.101	< 0.01	NS
Butyrate	0.01[c]	0.02[c]	0.01[c]	0.03[c]	0.09[b]	0.17[a]	0.029	< 0.01	NS
Hydrogen	-0.168	-0.325	-0.141	-0.527	-0.338	-0.309	0.182	NS	NS
Methane	0.00	0.00	0.00	0.02	0.07	0.06	0.033	NS	NS
Carbon Dioxide	0.21	0.49	0.25	0.48	0.26	0.22	0.193	NS	NS

[a - e] Means within a row not connected by the same letter are significantly different ($P < 0.05$).
[1] Interaction between the means of rumen fluid concentration treatment vs. Headspace H_2 (35.5 or 50%).
[2] Rumen fluid concentration was achieved by diluting processed rumen fluid with *in vitro* fermentation media. (Total = 10 mL)

Table 2.3: Effect of increasing incubation time on *in vitro* rates of formation of volatile fatty acid and fermentation gas in Experiment I.

	Incubation time (hrs)							H_2 x Time[1]
VFA	**0**	**1**	**3**	**6**	**12**	SEM	P-value	P-value
Acetate (mmol)	14.46^{c}	16.33^{b}	16.55^{ab}	16.98^{ab}	17.41^{a}	0.550	0.0024	NS
Propionate (mmol)	2.83^{b}	3.08^{b}	3.93^{a}	3.93^{a}	3.94^{a}	0.234	0.0004	NS
Butyrate (mmol)	0.58^{b}	0.62^{b}	1.01^{a}	1.04^{a}	1.06^{a}	0.083	< 0.0001	NS
Total VFA	17.87^{b}	20.03^{b}	21.49^{a}	21.95^{a}	22.41^{a}	0.826	0.0006	NS
A:P	5.07^{a}	5.22^{a}	4.40^{b}	4.56^{b}	4.80^{ab}	0.138	0.0005	NS
Hydrogen (mL)	7.39^{a}	3.11^{b}	2.90^{b}	1.13^{c}	1.49^{c}	0.322	< 0.0001	0.05[2]
Methane (mL)	0.02^{b}	0.06^{b}	0.38^{a}	0.29^{ab}	0.40^{a}	0.096	0.013	NS
Carbon Dioxide (mL)	10.21^{b}	14.46^{a}	14.70^{a}	16.71^{a}	14.55^{a}	0.714	< 0.0001	0.05
Total Gas Production	17.63	17.64	17.98	18.13	18.03	0.354	NS	NS
Rate of Formation (mmol Hour $^{-1}$)								
Acetate	-1.7e-16^{c}	0.99^{a}	0.70^{ab}	0.42^{abc}	0.25^{bc}	0.190	0.0104	NS
Propionate	-5.6e-17^{b}	0.07^{b}	0.37^{a}	0.18^{ab}	0.09^{b}	0.069	0.0056	NS
Butyrate	0.00^{c}	0.02^{c}	0.14^{a}	0.08^{b}	0.04^{bc}	0.021	0.0001	NS
Hydrogen	-1.1e-16^{a}	-1.5109^{b}	0.09^{a}	-0.2197^{a}	0.02^{a}	0.128	< 0.0001	NS
Methane	0.00^{b}	0.02^{a}	0.11^{b}	-0.01167^{b}	0.01^{b}	0.023	0.0057	NS
Carbon Dioxide	0.00^{b}	1.54^{a}	0.03^{b}	0.25^{b}	-0.1343^{b}	0.136^{b}	< 0.0001	NS

[a - e] Means within a row not connected by the same letter are significantly different ($P < 0.05$).
[1] The means for rumen fluid concentration treatment vs. Headspace H_2 (35.5 or 50%).
[2] The means for 10 mL H_2 was higher than the means for 6 ml headspace H_2 only for 0, 1 and 3 hrs of incubation.

Table 2.4: Main effect of headspace hydrogen and hay addition on the *in vitro* rates of formation of volatile fatty acid and fermentation gas in Experiment II

	Hay **				H_2 *			
VFA	**H**	**NH**	SEM	*P*-value	**35.5 %**	**50 %**	SEM	*P*-value
Acetate (mmol)	29.99	24.15	1.664	0.0003	27.46	26.67	1.586	NS
Propionate (mmol)	6.92	5.37	0.438	0.0002	6.22	6.07	0.417	NS
Butyrate (mmol)	1.81	1.43	0.112	0.0003	1.63	1.61	0.107	NS
Total VFA	38.72	30.95	0.042	0.0002	4.44	4.41	0.040	NS
A:P	4.35	4.50	0.042	0.0002	4.44	4.41	0.040	NS
Hydrogen (mL)	2.07	1.48	0.736	NS	1.26	2.29	0.702	0.0006
Methane (mL)	2.70	1.72	0.332	0.0009	1.84	2.57	0.317	NS
Carbon Dioxide (mL)	13.81	10.20	1.545	0.0038	12.29	11.72	1.473	NS
Total Gas Production	18.58	13.40	1.319	0.0001	15.40	16.58	1.257	0.05
Rate of Formation (mmol Hour $^{-1}$)								
Acetate	0.51	0.22	0.852	NS	0.22	0.52	0.812	NS
Propionate	0.20	0.12	0.341	NS	0.12	0.20	0.325	NS
Butyrate	0.12	0.10	0.184	NS	0.10	0.13	0.176	NS
Hydrogen	0.08	0.06	0.011	0.0068	0.05	0.09	0.010	NS
Methane	0.023	0.016	0.002	0.0003	0.017	0.023	0.002	NS
Carbon Dioxide	0.05	0.01	0.013	0.0006	0.03	0.04	0.012	NS

* The means for the effect of added Headspace H_2 %
** The means for the effect of addition of hay (H) or without hay (NH).
(All means across incubation time)

Table 2.5: Effect of increasing incubation time on the *in vitro* rates of formation of volatile fatty acid and fermentation gas in Experiment II.

	Incubation Time (hrs)						
VFA	**0**	**4**	**8**	**16**	**24**	SEM	P-value
Acetate (mmol)	25.04	24.98	26.89	28.14	30.30	1.586	NS
Propionate (mmol)	5.36^{c}	5.62^{bc}	6.12^{abc}	6.53^{ab}	7.09^{a}	0.417	0.08
Butyrate (mmol)	1.33^{c}	1.47^{bc}	1.61^{ab}	1.76^{a}	1.94^{a}	0.107	0.01
Total VFA	31.73	32.07	34.62	36.43	39.33	2.106	NS
A:P	4.67^{a}	4.45^{b}	4.40^{bc}	4.31^{c}	4.30b^{c}	0.040	0.0004
Hydrogen (mL)	9.06^{a}	-0.0377^{b}	-0.0413^{b}	-0.0447^{b}	-0.0698^{b}	0.702	0.0003
Methane (mL)	0^{c}	2.13^{b}	2.66^{ab}	3.26^{a}	3.07^{ab}	0.317	0.0008
Carbon Dioxide (mL)	8.01^{b}	11.16^{ab}	12.51^{ab}	13.86^{a}	14.50^{a}	1.473	0.0754
Total Gas Production	17	13.25	15.13	17.08	17.50	1.257	NS
Rate of Formation (mmol Hour $^{-1}$)							
Acetate	0	0.01	1.07	0.35	0.40	0.812	NS
Propionate	0	0.14	0.37	0.15	0.14	0.325	NS
Butyrate	0	0.22	0.18	0.09	0.07	0.176	NS
Hydrogen	0^{c}	0.14^{a}	0.10^{a}	0.05^{b}	0.05^{b}	0.010	0.0007
Methane	0^{c}	0.03^{a}	0.03^{a}	0.02^{b}	0.02^{b}	0.002	< 0.0001
Carbon Dioxide	0.00	0.05	0.05	0.03	0.04	0.012	NS

$^{a-e}$ Means within a row not connected by the same letter are significantly different ($P < 0.05$).

Table 2.6: Effect of increasing headspace hydrogen concentration on *in vitro* rumen fermentation parameters.

	Levels of Headspace H_2					
Fermentation Parameter	**5%**	**15%**	**30%**	**75%**	SEM	P-value
Acetate (mmol)	34.55	36.49	36.40	36.51	1.26	NS
Propionate (mmol)	7.43	7.81	8.35	8.51	0.67	NS
Butyrate (mmol)	1.74	1.90	2.51	2.03	0.28	NS
A:P	4.89	4.90	4.54	4.47	0.39	NS
Total VFA (mmol)	43.72	46.21	47.26	47.04	1.58	NS
Initial Hydrogen (mL)	15.82[c]	26.23[c]	44.61[b]	81.70[a]	6.55	< 0.0001
Final Hydrogen (mL)	1.78	8.26	19.50	19.81	6.78	NS
Methane (mL)	1.66[b]	2.18[b]	4.65[ab]	8.34[a]	1.42	0.008
Carbon Dioxide (mL)	131.08	127.64	112.01	119.46	8.64	NS
Total Gas Production (mL)	134.52	138.07	136.16	147.61	10.22	NS
Δ CH_4 (mmol)	0.01[b]	0.09[b]	0.22[ab]	0.41[a]	0.07	0.0004
Δ H_2 (mmol)	0.68[b]	0.77[b]	1.23[b]	3.08[a]	0.36	0.0002
CH_4/H_2 disappearance (mmol mmol^{-1})	-4.67[b]	3.17[ab]	12.29[ab]	18.90[a]	6.14	0.05

[a - e] Means within a row not connected by the same letter are significantly different ($P < 0.05$)
[*] $\Delta G_{Methanogenesis}$; Change in free energy for H_2 use in methanogenesis
[**] $\Delta G_{Acetogenesis}$; Change in free energy for H_2 use in acetogenesis through reductive acetogenesis

Table 27: Kinetic parameters for volatile fatty acid formation and methanogenesis in the presence of increasing headspace H_2

	Equation	R^2	Slope	Intercept y	Intercept x	V_{max} [a]	k_m [b]
Acetate	y = 0.0502x + 0.0861	0.926	0.0502	0.086	-1.715	11.614	0.583
Propionate	y = 0.1132x + 0.4409	0.995	0.1132	0.441	-3.895	2.268	0.257
Butyrate	y = 1.3106x + 0.9964	0.935	1.3106	0.996	-0.760	1.004	1.315
Methane							
Exp. - III**	y = -0.1716x + 1.5359	0.859	-0.1716	1.536	8.950	0.651	-0.112
Pre. 1	y = -0.1022x + 2.1571	0.522	-0.1022	2.157	21.107	0.464	-0.047
Pre. 2	y = 1.292x + 3.7407	0.445	1.2920	3.741	-2.895	0.267	0.345
Pre. 3	y = -0.3355x + 3.144	0.838	-0.3355	3.144	9.371	0.318	-0.107
CH_4 Overall Means	-	-	-	-	-	0.425 ± 0.172	0.0198 ± 0.22

[a] V_{max} = mmol hour^{-1}
[b] k_m = mmol of headspace H_2
** Denotes the kinetic parameters for Experiment – III and
(Pre.1) represents Preliminary Experiment 1(5, 15, 30 and 75% headspace H_2), 50:50 buffered rumen fluid, 1hr incubation. (Pre.2) represents Preliminary Experiment 2 (5, 15, 30 and 75% headspace H_2), 50:50 buffered rumen fluid, 1hr incubation. (Pre.3) represents Preliminary Experiment 3 (5, 15, 30 and 75% headspace H_2), 50:50 buffered rumen fluid, 4hr incubation.

Table 2.8: Standard change in Gibbs free energy (ΔG) for major fermentation pathways which influence fermentation profiles

ΔG (kJ)	Equation	Levels of Headspace H_2				SEM	P-value
		5%	**15%**	**30%**	**75%**		
$\Delta G_{Methane}$	$CO_2 + 4H_2 \rightarrow CH_4 + 2H_2O$	-105.32	-111.62	-115.60	-106.63	3.82	0.18
$\Delta G_{Acetate}$	$2CO_2 + 4H_2 \rightarrow CH_3COO^- + H^+ + 2H_2O$	-71.16[a]	-77.41[ab]	-81.03[b]	-74.09[a]	2.34	0.06
$\Delta G_{Propionate}$*	$CH_3COO^- + HCO_3 + H^+ + 3H_2 \rightarrow CH_3CH_2COO^- + 3H_2O$	-92.83[a]	-94.02[ab]	-94.82[ab]	-96.91[b]	1.07	0.05
$\Delta G_{Acetate}$*	$CH_3CH_2COO^- + 3H_2O \rightarrow CH_3COO^- + HCO_3 + H^+ + 3H_2$	92.83[a]	94.02[ab]	94.81[ab]	96.91[a]	1.07	0.05

The following ΔG° were used to calculate ΔG: Methane = -138.45 kJ, Acetate = -78.81 kJ, Acetate to propionate = -131 kJ[1] and Propionate to acetate = 131 kJ[1].
Assumptions pH = 6.6 [2], $10^{-6.6} = [H^+]$, $[HCO_3] = 7$ mol [2], $[H_2O] = 50$ mol [2]

[a - e] Means within a row not connected by the same letter are significantly different ($P < 0.05$).
[*] Represents the ΔG for interconversion of acetate to propionate ($\Delta G_{Propionate}$) and propionate to acetate ($\Delta G_{Acetate}$)
[1] (Van Lingen et al., 2016)
[2] (E M Ungerfeld & Kohn, 2006)

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Changing forage-to-concentrate ratio in the diet of dairy cows alters diurnal and weekly volatile fatty acid and fermentation gas profile *in vivo*

ABSTRACT

Increasing amounts of concentrates and easily fermentable starch in the diet of ruminants has been suggested as an affordable and readily applicable strategy for mitigating enteric methane emission in ruminant species. The objective of this study was to analyze the daily and hourly effects of two forage-to-concentrate diets on fermentation and rumen gas composition. Four lactating Holstein cows were used in a double switchback experimental design over 4 different 21 day feeding period (2 diets x 2 cows x 4 periods). The treatment diets were formulated by replacing forage of the diet (triticale and corn silage) with concentrate (ground corn and SBM). This resulted in a high forage (HF) 75:25 and a high concentrate diet (HC) 53:47 with forage to concentrate calculated on a DM basis. Each cow was fitted with an adapted cannula that facilitated rumen gas sampling without removing the whole cannula and risking air contamination. However, retrospective analysis suggests gas leaking around fistula. Treatment diet pairs received the opposing treatment diet before switching to the treatment diet on day 1 of each period. Body weight (BW), body condition score (BCS), Rumen VFA, rumen headspace gas, pH and Eh samples/measurement were collected four hours after feeding on day 1 of changing treatment diet. Subsequent samples were collected on day 8 and 21. Diurnal VFA, gas, pH and Eh samples were collected 1, 4 and 24 hours after feeding on a randomly selected day between day 21 and 28 of each period. Statistical analyses were done by JMP using a mixed model including fixed effects (diet, time and period) and the interactions of diet by time. Although cows on

the HC diet tended ($P = 0.08$) to eat less kg/day, they had greater ($P < 0.01$) milk yield (40.4 vs. 37.8 kg/day) and ECM/DMI (0.85 vs. 0.690kg of milk/kg of DMI). Acetate molar percentage tended ($P = 0.09$) to be higher in HF on day 8 and 24 with 53.9, 50.5 and 49.3 versus 52.3, 55.3 and 53.6 mM% for day 1,8 and 21 respectively. Methane rumen fraction in the HC was only numerically lower ($P = 0.25$) than that of the HF after 21 days with 12.9 and 17.7% methane for HC and HF respectively. Diurnal acetate molar % increased with time in both HF and HC diets, however, HF diets maintained a higher ($P < 0.01$) acetate concentration with 55.2, 53.5 and 60.8 % versus 48.3, 50.9 and 54.2% for HC diets. Similarly, rumen gas fractions from HF cows peaked 1 hour after feeding and 24 hours after feeding in HC cows. Additionally, the HF cows maintained higher CH_4 fraction thought the 24 hour period (26.1, 19.2 and 26.6 % versus 14.2, 12.8 and 22.5 % for HF versus HC respectively. Eh values were only different between day 1 and 8 of the period (-210.5 and -137.9 mV) as well as 1 and 4 hour after feeding (-249 and -178.3 mV). Changes in VFA and methane concentrations due to changing forage-to-concentrate ratio were greatest within 8 days, and decreased partially by 21 days. The VFA concentration peaked at 4 h after feeding, but CH_4 concentrations were lowest at that time across treatments.

INTRODUCTION

Enteric methane from ruminant species has been identified as one of the targets in reducing the anthropogenic sources of greenhouse gasses and improving animal feed efficiency (Haque, 2018; EPA, 2022). Enteric methanogenesis occur in the rumen as a valuable step involved in metabolizing the accumulated H_2 gas from the fermentation process and maintaining a healthy rumen ecosystem (Choudhury, Jena, et al., 2022). Additionally, methane production accounts for 3 – 5 % of dietary energy loss in ruminants resulting in costly declines in feed efficiencies (Cao, Liang, et al., 2016; Mengistu, Hendriks, et al., 2018; Judy, Bachman, et al., 2019). Numerous strategies have been tested of their effectiveness of reducing methane emission from dairy cattle. Some of these strategies include testing the effects of nitrates (Petersen, Hellwing, et al., 2015; Martinez-Fernandez, Denman, et al., 2016; Capelari, 2018), monensin (Montano, Manriquez, et al., 2015; Rosemond, 2021), magnesium (M. Wang, Wang, et al., 2016; Ma, Zhang, et al., 2019) and tannins (Witzig, Zeder, et al., 2018) among others. These additives have showed varying results and coupled with other additive effects on VFA production, methane inhibition, DMI, rumen pH, DM digestibility with very little consistencies.

Altering the forage to concentrate ratio in the diet of ruminants has also been widely investigated for its effectiveness on decreasing enteric methane emissions while simultaneously improving other production parameters of ruminants. Some major observations from these experiments highlight the relationship between higher inclusion rates of nonstructural carbohydrates or concentrate in ruminant diets and decreased A:P ratio and methane emissions along with improved production and feed efficiency.

Aguerre and Wattiaux et al. (2011) increased the forage level from 47 to 68% of TMR DM and tried to use changes in the VFA composition to predict the methane production but were unsuccessful. A study testing the *in vivo* effect of low (33%) and high (65%) concentrate diets reported significantly higher ($P = 0.02$) methane yields of 492 and 404 g/d for the low versus high concentrate diet respectively. Similar trends were reported for milk efficiencies (methane %/GE intake) and CO_2 yields in g/day (Bayat, Ventto, Kairenius, Stefanski, et al., 2017).

Increased amounts of concentrate in ruminant diets reduces the ruminal pH which also has negative effects on the animal health and overall digestibility in the reticulo-rumen. This lower rumen pH is associated with increased concentration of lactic acid due to increased amounts of concentrate in the diet. Propionate synthesis mainly occurs via the fumarate-succinate pathway (Bharanidharan, Arokiyaraj, et al., 2018; Xue, Chen, et al., 2021), however increasing concentrate inclusion has demonstrated that an additional propionate synthesizing pathway; the acrylate or lactate pathway is always active (Gozho, Krause, et al., 2006; Choudhury, 2022). Among other factors, differing growth rates of fiber digesters versus the starch digesters in the rumen may also result in variation in fermentation profile depending on the hours after feeding (Lechartier & Peyraud, 2010; Olijhoek, Hellwing, et al., 2016).

Very few *in vivo* studies have investigated the dietary effects of feeding two different forage to concentrate ratio diets and its effects changes in both VFA profile and rumen headspace gas profiles as the rumen adjust to the diet. Therefore, this study aimed to identify periodical and diurnal changes in VFA concentration and rumen headspace gas composition of cows fed two different F:C diets. It was hypothesized that cows adjusted to the HC diet would have greater propionate with lower methane proportions compared to the HF cows. Additionally, hydrogen and propionate proportions were hypothesized to be greatest between 1 and 4 hours of feeding.

MATERIALS AND METHODS

All experimental procedures were approved by the University of Maryland Institutional Animal Care and Use Committee prior to the start of the feeding experiment (IACUC: Protocol number 1376980-2).

Animal Housing and Diet

Four multiparous Holstein Friesian cows (1565 ± 29.2 lbs.) were assigned to one of 2 diet treatments and housed in a freestall dairy barn fitted with individual Calan gates. Prior to the start of the experiment, experimental animals were fed from individually assigned Calan gates for a three-week feeding adjustment period. For the remainder of the study cows were fed two modified total mixed rations (TMR) formulated to represent the treatment diets and meet the NRC requirement for lactating dairy cows with daily feed adjusted to allow a 10% refusal. Feed and water were offered ad libitum and the experimental animals remained a part of the farm's regular milking schedule. An additional replacement cow was also housed with the experimental animals, bringing the total to five lactating cannulated cows. The replacement cow received the basal diet offered to the rest of the milking herd for the duration of the study unless a replacement cow was needed.

The normal TMR offered to the milking herd was altered to create two different treatment diets for the experiment. Altering the concentrate (soybean meal and ground corn) and forage (triticale and corn silage) components of the basal diet yielded a high-forage (HF) and high-concentrate (HC) diet (Table 3.1). The forage-to-concentrate ratio was 75:25 and 53:47 (%DM) for the HF and HC diet respectively. Both treatment diets were isocaloric and nitrogenous while maintaining the recommended NDF ADF and NFC NRC requirements for lactating dairy cows (National Research Council, 2003).

Experimental Design

Two pairs of cows (n=4) were assigned to one of two treatment diets and used in a double switchback experimental design. Each pair of cows alternated between treatment diets for a total of 4 experimental periods. Therefore, each cow received each diet twice throughout the experiment with a total of 16 experimental units (2 diets x 2 cow x 4 period). A 21-day diet adjustment period allowed each feeding pair (cows on similar treatment diet) to adjust to the opposing treatment diet for period-1 and facilitated a diet switch simulation on day-1 of the feeding experiment (Figure 3.1). The four feed experiment periods aligned with various physiological states in the lifecycle of a dairy cow that may be grouped in two distinct phases of metabolic demands. Periods 1 and 2 occurred during the antepartum and early lactation period respectively, while periods 4 and 5 both occurred during mid-lactation. Various samples were collected 4 hours post morning feeding on days 1, 8 and 21 of each period to assess changes that occur in the rumen as cows switch and adjust to each treatment diet (within period changes). Additional samples will be collected 1 and 24 hours after feeding on day 21 of each period to access the daily changes that occur in the rumen post morning feeding (diurnal changes).

Animal and Rumen Performance Measurements

Individual feed intake was recorded using the feed offered and feed refusal measurements from a data ranger feed mixer/scale. TMR was prepared fresh daily and samples of the TMR, ground corn, soybean meal, corn silage and triticale were collected on a weekly basis for composite TMR and individual ingredient nutrient analysis at the end of each period (wet chemistry analysis - Cumberland Valley Analytical Services, Waynesboro PA). Body weight (BW), body condition score (BCS), rumen pH, rumen redox potential (Eh), rumen gas fractions and volatile fatty acid concentration (VFA) were sampled 4 hours after feeding for within period changes (days 1, 8, and 21). Additional rumen gas, VFA, pH and Eh samples were collected 1 and 24 hours after feeding to assess the diurnal changes occurring in the rumen.

Rumen Gas Sampling

Prior to the start of the feeding experiment each cow's rumen cannula was replaced with a modified rumen canula designed to facilitate the collection of rumen gas sample without opening the rumen cannula. Each cannula was fitted with a hose barb (Everbilt ¼ inch barb x ¼ inch MIP brass adapter fitting) and fastened in place on both side on the cannula using a steel washer (Everbilt ¼ stainless steel flat washer) coupled with a rubber gasket. Tubing (< 2-inches) was secured to the brass barb with a hose clamp and a two-way Luer-lock stopcock valve attached to the free end of the hose facilitated gas collection with a syringe. An additional Luer lock twist cap plug was fastened to the stopcock to prevent rumen gas leaks if the stopcock opened accidentally. All tubing and valve connections were sealed with epoxy to prevent leakage.

At the time of gas collection, cows were restrained in a chute and a 30 mL syringe (fitted with a two-way stopcock) was attached to the two-way Luer-lock stopcock end of the cannula hose. Each syringe was flushed twice with rumen gas before 15-20 mL of rumen gas was sampled. Both stopcocks were closed before sampling syringe was removed and stored for gas analysis within 24-hours of collection.

Rumen Gas Analysis

Gas fractions were measured using gas chromatography (Hewlett-Packard model 6890) with a 15.0 ft length × 1/8 in. outer diameter × 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). Argon was used as a carrier gas with a constant pressure at 35.0 psi, flow of 6.5 mL/min. The column remained isothermal 150°C and held for 9.5 min. Detector was set to 250°C with a reference flow of 56.0 mL/min and makeup gas (Helium) set to 7.0 mL/min. Gas measurements were reported as gas fraction estimates obtained from standard curves.

Rumen pH and Eh Samples

Rumen pH was measured immediately after opening the cannula using a portable pH meter (FiveGo pH meter F2) fitted with a pH electrode. An additional portable pH meter, fitted with an oxidation-reduction potential (ORP) probe (Mettler Toledo ORP electrode Inlab Redox Ag) was used to immediately measure the Eh. An Erlenmeyer flask was filled with ~150mL of rumen fluid, the ORP inserted, and the flask stopped to reduce air exchange for Eh measurement. Each Eh reading took approximately 10 – 15 minutes to achieve a stable reading.

VFA collection and analysis

Approximately 2mL of rumen fluid were collected from the rumen at each sampling timepoint and stored at -20°C for later VFA analysis. The VFA samples were prepared using a modified Erwin et al. (1961) method. Samples were thawed at room temperature then spun in a centrifuge at 12,000 × g for 30 minutes at 4°C. 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 1.8 m length x ¼ in. outer diameter x 4 mm inner diameter packed GC column (60/80 Carbopack-11766 support, model 1-21641, 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 29 mL/min. The initial column temperature was 110°C held for 1 min, then increased to 180 °C (ramp of 10°C/min) for 1 min, then increased to 200 °C (ramp of 20°C/min) for 3 min, and a post-run temperature of

110°C. The detector temperature was 250°C with a hydrogen and air flow of 30 mL/min and 200 mL/min, respectively.

Statistical Analysis

Statistical analyses were conducted using JMP Pro 15 (JMP®, Version 15.2.0 SAS Institute Inc., Cary, NC, 1989-2007). The following model statement was used for statistical analysis of the within period changes: $Y_{ijk} = m + TD_i + D_j + P_k + (TD \times D)_{ij} + e_{ijk}$ where TD, D and P represented the fixed effects of treatment diet, day of period and period respectively. An additional model statement, $Y_{ijk} = m + TD_i + T_j + P_k + (TD \times T)_{ij} + e_{ijk}$ where TD, T and P represented the fixed effects of treatment diet, time of day and period respectively, tested the diurnal effects on multiple samples taken on day 21 of each period. Statistical differences were accepted at $P < 0.05$ and a tendency toward statistical difference was declared at $P > 0.05$ but < 0.10.

RESULTS

Wet chemistry analysis of the composite samples for the 4 periods showed that the HF diet had significantly higher ($P < 0.05$) fiber content with ADF measurements of 22.9 and 16.05 % DM and NDF 35.9 and 26.88 % DM for HF and HC diets respectively. Nutrient compositions analysis also highlighted the differences in the energy content in both diets. The HC diet had significantly greater ($P < 0.05$) metabolizable energy (HC: 1.33 and HF: 1.26 Mcal/lbs). Similar trends were observed with the high concentrate diet having higher ($P < 0.05$) estimates for net energy of lactation, maintenance and gain (Table 3.2). High concentrate diets also had a tendency (*P* = 0.07) to be higher in crude protein content (HC: 15.35 and HF: 14.48 % DM) and phosphorus levels. Overall, the diets were similar between all 4 different periods except for copper which was significantly higher (*P* = 0.03) for period 2. Copper level for periods 1, 3, and 4 ranged from 10 to 12.5 ppm while the levels for period 2 were approximately 19 ppm.

Period Effects

There was no difference (*P* < 0.05) in the mean body weight (kg) or body weight change (kg/d) between the 4 feeding periods. However, mean body condition scores were significantly lower (*P* < 0.05) in period 4 compared to periods 1, 2, and 3 (Table 3.3). Additionally, feed intake on an as-fed basis (kg/d), DMI (kg/d), milk production (kg/d), ECM (kg/d and ECM/DMI were all significantly lower (*P* < 0.05) in period 1 (peripartum) and peaked during period 2 (early lactation; Table 3.3).

There was no difference (*P* < 0.05) observed between dietary treatments for either body weights, weight change or BCS. Feed intake on an as-fed basis tended (*P* = 0.08) to be greater for cows on the HF diet compared to intake of cows on the HC diet, with mean intake of 56.2 and 54.5 kg/d for HF and HC respectively. However, cows on the HC diet had significantly greater (*P* ≤ 0.01) dry matter intake (kg/d) and had greater milk production in all periods of the experiment. Cows fed the HC diet produced an average of 40.4 kg/d of milk and 0.85 ECM/DMI versus 37.8 kg/d of milk and 0.69 ECM/DMI for cows on the HF diet.

Total VFA concentration (mM) as well as propionate and butyrate concentrations (mM) were highest (*P* ≤ 0.01) for period 2 and 4 of the experiment, however acetate concentration was similar (P = 0.24) between all feeding periods (Table 3.4). Propionate mM % increased ($P \leq 0.01$) as cows progressed through consecutive feeding period while butyrate mM% decreased. Rumen propionate mM% was 22.8 % in period 1 and increased to 37.2, 37.4 and 37.3 % for periods 2, 3, and 4 respectively, while butyrate mM% were 17.7, 5.1, 2.9 and 3.1 for periods 1, 2, 3, and 4 respectively. Consequently, the mM% for acetate decreased significantly ($P < 0.05$) between each feeding period (Table 3.4).

Rumen hydrogen composition tended to be higher (P = 0.10) for periods 1 and 2 with mean H_2 fractions of 7.0 and 5.1% respectively, while periods 3 and 4 mean hydrogen fraction remained below 3.1% of the total rumen gas. Conversely, the rumen methane fractions were highest ($P < 0.05$) for periods 3 and 4 with CH_4 % reaching highs of 28.8 and 24.5 % of the rumen gas respectively. Carbon dioxide fractions measured directly from the rumen also varied significantly ($P < 0.05$) between periods with mean CO_2 representing 75.9, 79.2 68.3 and 72.3 % of the total gas collected from the rumen for periods 1, 2, 3, and 4 respectively (Table 3.4).

Within Period Effects

Acetate molar percentage (mM %) tended (P = 0.09) to have a day by diet interaction, with acetate mM % being significantly higher in cows fed a HF diet only on day8 and 21 of the feeding experiment, there was no differences in acetate mM% on day 1 of the experiment. Acetate molar percentage decreased from 53.9 to 50.5 and 49.3 % in cows fed a HC diet while HF acetate percentage increased from 52.3 to 55.3 and 53.6 % for days 1, 8, 21 (Figure 3.2-A). Similarly, propionate molar percentage had significant day by diet interaction (P = 0.04), with HF cows being significantly higher on day 1 (HF:34.8% and HC:31.8%) and HC having higher propionate molar percentage on days 8 (HF:31.5% and HC:34.8%) and 21 (HF:31.6% and HC:33.6%) (Figure 3.2-B). Forage to concentrate ratio had no dietary effect (P = 0.45) on butyrate mM% however, there was a tendency (P = 0.07) for butyrate mM% to be higher on day 21 of the feed experiment (Figure 3.2-C).

Hydrogen fraction tended (P = 0.06) to be higher in the HF diet with 9.15, 5.3 and 6.5 % H_2 versus 4.8, 4.8 and 5.5 % H_2 for HC diet on day 1,8 and 21, respectively (Figure 3.4-A). Rumen methane fractions were similar between the HC and HF diets on days 1, 8 and 21 of the feed experiment. Treatment diet had no effect (P = 0.25) on the CH_4 % in the current study, however CH_4 % transition from numerically higher CH_4 % in HC versus HF on day 1 (HC:23.7 and HF:19.3%) to lower CH_4 % on day 21 (HC:12.9 and HF:17.7%) (Figure 3.4-B). Carbon dioxide fraction of rumen gas was similar between treatment diets (P = 0.99), however, CO_2 % tended (P = 0.09) to be higher on day 21compared to day 1 and 8. Carbon dioxide fraction was 71.5 and 71.50 % of day 1, 71.7 and 72.1% on day 8 and increased to 81.6 and 75.8% on day 21 for HC and HF diets respectively (Figure 3.4-C).

Diet had no effect of rumen pH (P = 0.63), with mean pH readings of 5.93 and 5.79 for HC and HF diets respectively. Similarly, diet had no effect on rumen redox potential (P = 0.65 with mean ORP reading of -158.44 and -192.77 mV for cows on the HC and HF diet respectively (Figure 3.5). Additionally, the rumen pH stayed constant throughout the whole period with probe readings of 6.01, 5.60 and 5.96 ± 0.27 on days 1, 8, and 21. However, redox/ORP measurements had quadratic response (P =

0.0095) between day 1, 8 and 21 with ORP readings of -211.7, -135.60 and -160.33 mV respectively (Figure 3.5).

Diurnal Changes

Overall acetate molar percentages were significantly (time *P* = 0.002) higher 24 hours after feeding compared to earlier time points (1 and 4 hours post feeding). Additionally, acetate molar percentages were significantly higher (diet effect *P* = 0.008) in the rumen on HF cows (55.2, 53.5 and 60.8 %) compared to the HC cows (48.3, 50.9 and 54.2) at 1, 4 and 14 hours after feeding (Figure 3.6-A). There was no significant effect of time (*P* = 0.14) or diet (*P* = 0.21) on the propionate molar fraction in the feed experiment. However, there was a diet effect (*P* = 0.002) on butyrate molar percentages. Cows fed a HC diet produced more butyrate (16.07 mM%) compared to those adjusted to a HF Diet (12.72 mM%). Butyrate molar percentage also appeared to be significantly lower (*P* = 0.002) 24 hours after feeding compared to the 1 and 4 hours post feeding (Figure 3.6-C). Total VFA production also peaked (*P* = 0.006) 4 hours after feeding with mean ruminal VFA concentrations between diets ranging from 87.44, 95.02 and 73.11 mM for samples collected 1, 4 and 24 hours post feeding. Treatment diet had no effect on the total VFA concentrations (*P* = 0.89).

Rumen H_2 percentage tended (*P* = 0.06) to be higher in cows fed the HC diet, however there was no time effect (*P* = 0.21) on diurnal H_2 percentage. Methane was significantly (*P* = 0.05) greater in cows adjusted to the HF (26.1, 19.2 and 26.6 %) compared to cows adjusted to the HC diet (14.2, 12.8 and 22.5 %) for 1, 4 and 24 hours post feeding (Figure 3.7-B). Rumen CO_2 molar percent was not different between treatment diets 1, 4, or 24 hours after feeding (P = 0.77), however CO_2 percentage tended (P = 0.06) peaked (HC: 83.4% and HF: 75.5%) for both diets 4 hours after feeding compared to 69.8 and 68.1% at 1 hour after feeding and 71.0 and 69.6% 24 hours after feeding for HC and HF diet respectively (Figure 3.7-C).

Treatment diet had no effect on rumen pH (P = 0.45) or the oxidation-reduction potential of the rumen (P = 0.11). However, rumen pH increased (P < 0.0001) to 6.49 24 hours after feeding compared to samples collected 1 and 4 hours after feeding (6.01 and 5.99, respectively) (Figure 3.5). Conversely, ORP readings were only significantly different (P = 0.05) between the samples collected 1 and 4 hours after feeding with mean redox readings of -249.4, -178.3 and -209.5 mV for 1, 4 and 24 hours post feeding (Figure 3.5).

DISCUSSION

Observed period difference in intake and milk production were a result of the physiological differences and differences in metabolic demands. All intake and milk parameters were lowest in period 1 of the diet due to cows all being ≥ 250 days in milk at the start of the study. Subsequently all milk and intake parameters increased during period 2 of the switchback design.

Increasing the forage to concentrate ration in the diet of ruminants is generally associated increased bulk in the rumen and typically has a negative effect on the feed intake (Aguerre, 2011; Bayat, Ventto, Kairenius, Stefański, et al., 2017). Similarly, increased NDF and ADF present in the HF diet decreased DMI in the current study with cows adjusted to the HC diets. Similar results were also observed a previous studies where increasing inclusion of concentrate in the TMR of dairy cows improved DMI (Bayat, 2017; Yang, Ferreira, et al., 2018; Ben Meir, Nikbachat, et al., 2021). Other factors such as chemostatic or metabolic regulation and rumen distension or gut fill have also been identified as key mechanisms regulating DMI (Allen, Sousa, et al., 2019; Ben Meir, 2021). Therefore, while the current study aimed to identify influence of HC and HF diets on rumen fermentation, both diets were limited by intake. HF cow intake may have been limited by rumen fill and distension, however the higher CP content in the HC diet may have influenced greater feed intake. Studies have shown increased DMI to be positively associated to CP content in the TMR of lactating cows and the increased RDP effects on improving feed digestibility has been identified and probable cause (Oldham, 1984). Additionally, the HC TMR in the current study had greater NE_L, NE_M and NE_G Mcal/lb compared to cows on the HF diet (Table 3.2), suggesting that HC diet may have been also regulated by the energy requirement rather than rumen fill. Studies conducted by Allen (2000) suggested that in addition to rumen fill and distention the energy requirement of a diet may also influence DMI. Therefore, the tendency for higher CP content in the HC diet coupled with higher probability of rumen distension from rumen fill in HF may have resulted in the dietary differences in intake.

Increased milk yield (kg/d) observed in the HC treatment diet supports previous understandings of higher inclusion rates of concentrate in the TMR of dairy cows resulting in higher milk yields. This is partly due to the increased NFC content associated with increase concentrate in HC diets. Similar finding were reported in other studies conducted by Arriola and company (2011) showed that milk yield increased from 31.9 to 33.6 kg/d by increasing forage-to-concentrate ration from 67:33 to 52:48 respectively. Additional studies where HF and HC diets with similar NDF content (HF-NDF: 34.3 and FC-NDF: 29.0 %DM) were fed to dairy cows tended to decrease milk yield from 44.4 to 42.2 %DM for HF and HC diet respectively.

Within Period Effects

Acetate molar percentages decreased as the rumen environment adjusted to the HC while cows on the HF diet had increased in acetate molar percentage as they adjusted to their treatment diet. Additionally, total VFA production decreased in the cows fed the HF diet (HC: 96.15 mM and HF: 86.99 mM).These findings support the understanding that a rumen condition adjusted to HF diets favors the production of acetate and while HC-adapted rumen condition favor propionate production (Aguerre, 2011; Arriola, 2011). Previous studies assessing the effects of two different F:C silage and hay ratios on rumen fermentation showed cows fed HF hay and silage had 64.4 and 63.3 acetate mM%, compared to 61.5 and 61.6% acetate in HC diets replacing hay or silage for concentrate (Murphy, Åkerlind, et al., 2000). Gradual adaptation of the rumen environment to diets containing higher levels of nonstructural carbohydrates promote the growth of lactate utilizing bacteria in the rumen and subsequent increased propionate production via the acrylate pathway (Gozho, 2006; L. Wang, Zhang, et al., 2020). As a result of the switches in VFA composition between treatment diets the A:P ratio for HF cows increased while HC A:P ratio decreased as the rumen adapted to their respective diets (Figure 3.3-A). Wang et al., (2020) decreased ($P < 0.01$) A:P ratio and total VFA (mM) in rumen samples collected 4 hours after feeding Holsteins fed HC (1.91 and 160.90) versus HF (4.67 and 149.12, respectively) diets.

Measuring hydrogen composition in the rumen is rarely reported due to the fact that the gas is highly elusive and rarely captured in fermentation gas sampling, partly due to the high rates of H_2 metabolism and low concentrations in the rumen (Smolenski & Robinson, 1988; Lunda & Yang, 2014). Overall hydrogen percentages in the current study (HF: 6.7% and HC: 4.0%) were markedly higher than reported H_2 composition in other studies. Numerous studies have reported low to negligible hydrogen composition ranging from 0.1 to 1% in the rumen (Barry, Thompson, & Armstrong, 1977; Hegarty & Gerdes, 1999). More recent studies by (Olijhoek, 2016) reported H_2 rumen gas composition ranging from 0.17 – 0.59 when calculating the H_2 % from the total of H_2, CH_4 and CO_2 emissions (L/day). Similarly, the rumen gas sampled in the present experiment was presented as a percentage the total H_2, CH_4 and CO_2 fraction only. Rumen gas samples collected at the same timepoint with recorded air fractions > 50% were discarded and the sample collection repeated the following day to ensure each *in vivo* gas sample had very little contamination by air leakage. Additionally, the adapted rumen cannulas used in the study were tested in 2 separate canulated cows where rumen gas was collected 4 hours after feeding over a 3-month period and the rumen gas was analyzed within 30 minutes of sample collection. This 3-month period tested the modified device for air leakage and the recorded H_2 % were similar to those recorded in the *in vivo* study. Acetogenesis is a major contributor to ruminal hydrogen concentrations (Janssen, 2010; Ungerfeld, 2013). Therefore, excess hydrogen from HF cows in the present study may be explained by acetogenic rates.

Increased acetate mM% in HF cows between day 1 versus day 8 and 24 may have increased availability of rumen H_2 which was quickly reduced methane by hydrogenotrophic methanogens. Decreased propionate molar % resulted from an increase ($P < 0.01$) in acetate mM (41.5, 45.9 and 48.2 mM for 1, 8 and 21 days respectively) rather than an increase in propionate production (28.4, 26.4 and 29.6 mM for 1, 8 and 21 days respectively). Acetate concentration decreases in the rumen of cows as they adapt to the HC diet and would decrease the availability of H_2 for hydrogenotrophic methanogens lowering methane percentages. Other studies have shown that increasing the availability of nonstructural carbohydrates in the diet of ruminants changes the rumen microbiome by increasing the abundance of lactate utilizing bacteria which increases feasible pathways for propionate production (Gozho, 2006; L. Wang, 2020).

Diurnal Changes

Differences in the diurnal acetate and propionate molar percentages between treatment diets were mostly observed at 1 versus 24 hours after feeding only for HF rations (Figure 3.3). These diurnal changes in int VFA mM% and consequently the A:P ration were driven mainly by low propionate concentrations in HF cows 24 hours after feeding and high acetate in the HF cows at 1 and 4 hours after feeding (Figure 3.6). Total VFA concentration in the current study was significantly higher ($P < 0.01$) 4-hours after feeding with rumen concentrations of 87.44, 95.10 and 73.11 for 1, 4 and 14 hours post feeding, respectively. These findings are in accordance with research conducted by Gozho et al. (2006) who found that total VFA concentration (collected

1, 4, 7 and 21 hours after feeding) was significantly higher at the 4-Hours post feeding timepoint and lowest at 21 hours post feeding. The CO_2 and CH_4 fraction of rumen gas was lowest 1 and 4 hours after feeding in the present study (Figure 3.7), similar trends were seen in earlier studies characterizing the *in vivo* profile of rumen gas for ruminants on a hay or grain diet (Barry, Thompson, et al., 1977b) The study found a marked decrease in the CO_2 and CH_4 fraction 2 hours after each feeding additionally the decrease was greater for rumen adapted to a high concentrate diet compared to that of a HF diet. The ratio of CO_2:CH_4 fraction in the HC diet was 4.9, 6.5 and 3.2 while the HF diet ratio was 2.6, 4.0 and 2.6% for 1-, 4- and 24-hour post feeding. The observed heightened CO_2:CH_4 ratio at 4 hours after feeding may be explained by the faster rate of CO_2 formation in the rumen compared to the rate of formation H_2 to serve as a reducing for CH_4 production.

Changes in Rumen Redox Potential

The redox potential in the current study was relative to the half-reaction on hydrogen, however, changes in rumen pH (H^+) affect the Eh and partial pressure of hydrogen (P_{H2})(Hegarty, 1999). The Nernst Equation [eq. 1] is frequently modified [eq. 2] to express the relation between these fluctuating parameters (Sauer & Teather, 1987; Hegarty, 1999).

$$E_h = -E_0 - RT/F \ln ([H^+] / [H_2]) \qquad \text{[eq. 1]}$$

$$E_h = 0.062 * \log [H^+] / P_{H2} \qquad \text{[eq. 2]}$$

In the current study there were significant (diurnal: P < 0.01) and numeric (within period) fluctuations in the rumen pH (Figure 3.5 bottom right and left) warranting the correcting of the oxidation potential reading (E_0; Figure 3.5 top right and left) using equation 2. The corrected Eh readings were 0.66, 1.63 and 1.01 for 1, 8 and 21 days on treatment diet while diurnal corrected values were Eh 0.44, 1.01 and 0.96 for 1, 4 and 24 hours after feeding. The sharp decline in Eh corrected values (rH) readings after 2 hours feeding has also been reported in previous studies (Barry, 1977b; Lund, Maigaard, et al., 2021). Diet had no effect on diurnal or within period (day 1,8 and 21) Eh or pH in the current study, however, studies testing the effect of grain versus non-grain diet did result in lower redox potential and higher pH in the rumen of low grain diets (Julien, Marden, et al., 2010; Friedman, Shriker, et al., 2017). The lack of observed difference of diet or time effect in the current study may be partly due to the high variability reporting in redox reading from the rumen and the reading that may have not have fully stabilized. Samples in the current study were all collected and allowed to stabilize for 15-20 minutes before the reading were recorded; however, literature appears to suggest that rumen fluid may require up to 45 minutes before a stable reading is achieved.

CONCLUSION

Increasing the forage-to-concentrate ratio in the diet of cows increased the acetate and decreased the propionate VFA fractions while higher concentrate inclusion increases propionate and decreased acetate fractions in the rumen. Therefore, confirming the influence of higher dietary concentrate inclusion on shifting the VFA profiles in favor of lower A:P ratio. While methane overall composition decreased in both diets by day 21 of the period, cows on HC diets saw the most reduction in methane production. Additionally, rumen CO_2 accumulated when methanogenesis lowest. Diurnal changes in rumen VFA showed that acetogenic composition peaked closer toward 24 hours after feeding while butyrate and propionate production was more active 1 and 4 hours after feeding. Similarly, methane composition peaked 24 hours after feeding and occurred while acetate composition was at its highest but after CO_2 accumulation. These results highlight the dependency of rumen methanogenesis on the acetogenic process to provide H_2 as reducing equivalent and CO_2 as an oxidizing agent. Additionally, HC diets consistently produced more propionate and less methane after 1 hour on the diet.

Table 3.1: Treatment Diet ingredients and formulation**.**

Feed Ingredients	Diets		
	Basal[a] (%)	High-Forage (HF) Diet (%)	High-Concentrate (HC) Diet (%)
Corn Grain Ground[c]	8.43	3.21	17.45
Soybean Meal 48%[c]	4.51	4.83	7.27
Lactating Mineral	5.50	2.17	1.19
Megalac[b]	0.23	1.19	0.54
Triticale Silage[d]	25.81	36.91	29.89
Corn Silage[d]	55.52	51.69	43.66
F:C (as fed)	86:14	92:8	75:25
F:C (Dry Matter)	-	75:25	53:47
TOTAL	100.000	100.000	100.000

Each cow receives approx. 120 lbs. cow^{-1} day^{-1}

[a] Refers to the normal diet formulation of the normal diet offered to lactating cows at the Clarkesville Research Facility

[b] High-energy rumen-protected fat source included in diet

[c] Concentrate portion of the diet to be altered

[d] Forage portion of the diet to be altered

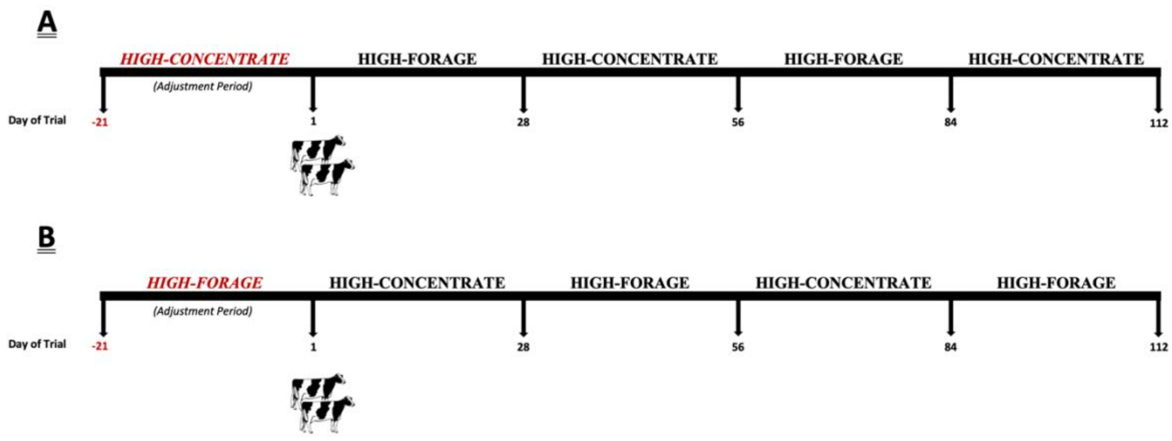


Figure 3.1: Feeding experiment timeline and treatment diet assignment to each pair of cows (A or B) in a four-period double switchback experimental design.

Table 3.2: Nutrient composition analysis of treatment diets.

	Diets				
	High-Forage (HF)	High-Concentrate (HC)	SEM	P-Value	Period Effect
Nutrient Composition (% DM)					
Crude Protein	14.48	15.35	0.35	0.07	0.22
Soluble Protein	7.60	6.60	0.73	0.23	0.12
ADF	22.9	16.05	2.13	0.04	0.53
NDF	35.9	26.88	2.23	0.02	0.25
NE_L (Mcal/lbs)	0.76	0.79	0.012	0.04	0.66
NE_M (Mcal/lbs)	0.84	0.9	0.01	0.01	0.36
NE_G (Mcal/lbs)	0.55	0.6	0.01	0.04	0.63
ME (Mcal/lbs)	1.26	1.33	0.02	0.02	0.51
Ash	7.00	6.61	0.3	0.23	0.07
Calcium	0.72	0.52	0.06	0.07	0.08
Phosphorus	0.34	0.37	0.01	0.07	0.5
Magnesium	0.21	0.2	0.01	0.21	0.11
Potassium	1.73	1.49	0.13	0.14	0.84
Sodium	0.26	0.17	0.04	0.08	0.05
Iron (ppm)	245.8	211.8	22.5	0.19	0.17
Manganese (ppm)	59.3	48.3	6.3	0.15	0.18
Zinc (ppm)	53.3	45.3	4.6	0.15	0.14
Copper (ppm)	14.5	12	1.2	0.1	0.03

ADF: acid detergent fiber, NDF: adjusted neutral detergent fiber, NE_L: net energy of lactation, NE_M: net energy of maintenance, NE_G: net energy of gain,

Table 3.3: Differences in animal performance and milk parameters between feeding periods and treatment diets.

	Period						Diet			
	1	**2**	**3**	**4**	SEM	P-value	**High Conc.**	**High Forage**	SEM	P-value
Performance										
BW (kg)	739.3	697.6	713.3	723.6	16.3	N.S.	717.7	711.5	11.40	N.S.
BW Change (kg/d)	-2.9	1.1	1.3	1.3	1.8	N.S.	0.9	0.3	1.24	N.S.
BCS	3.4 [a]	3.4 [a]	3.5 [a]	3.1 [b]	0.1	<0.01	3.3	3.4	0.04	N.S.
Feed Intake - As Fed (kg/d)	53.2 [a]	56.7 [b]	55.8 [b]	56.1 [b]	0.9	0.01	54.5	56.2	0.7	0.08
DMI (kg/d)	23.4 [c]	24.8 [ab]	24.5 [b]	25.9 [a]	0.4	0.002	24.7	24.2	0.3	<0.01
Milk Yield (kg/d)	25.1 [d]	46.7 [a]	44.3 [b]	40.0 [c]	0.7	<0.01	40.4	37.8	0.5	<0.01
ECM (kg/d) *	29.6 [d]	48.8 [a]	46.3 [b]	41.7 [c]	0.7	<0.01	42.1	41.5	0.4	N.S.
ECM/DMI **	0.6 [c]	0.9 [a]	0.9 [a]	0.7 [b]	0.01	<0.01	0.85	0.69	0.01	<0.01

[a–d] Means in the same row with different superscripts differ ($P < 0.05$).

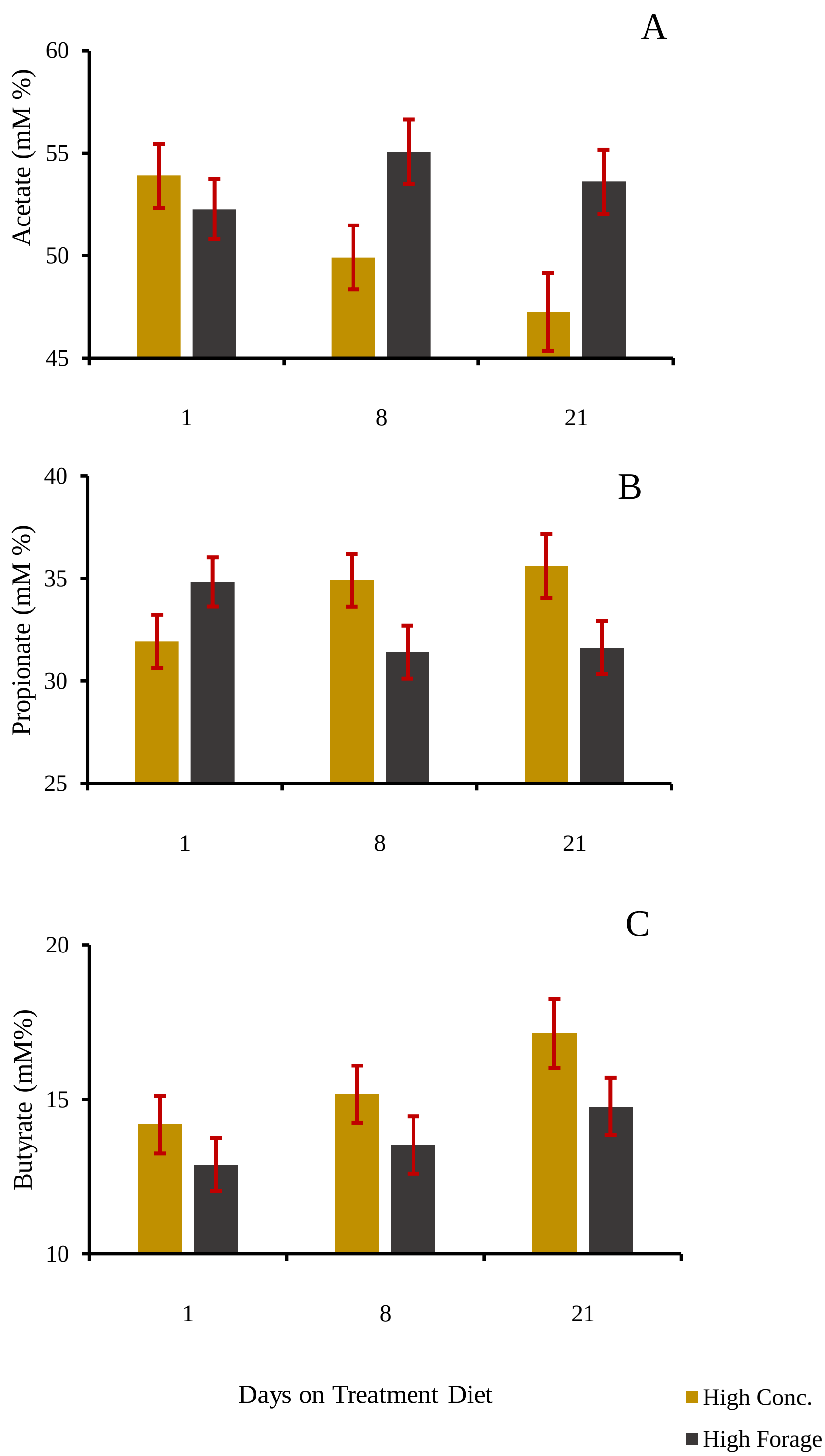
* Energy corrected milk (ECM) calculated as milk = (0.327 x milk in kg) + (12.97 x fat in kg) + (7.2 protein in kg).

** Efficiency calculated as ECM (kg/d) divided by DMI (kg/d)

Table 3.4: Differences in rumen volatile fatty acid and gas composition between feeding periods and treatment diets.

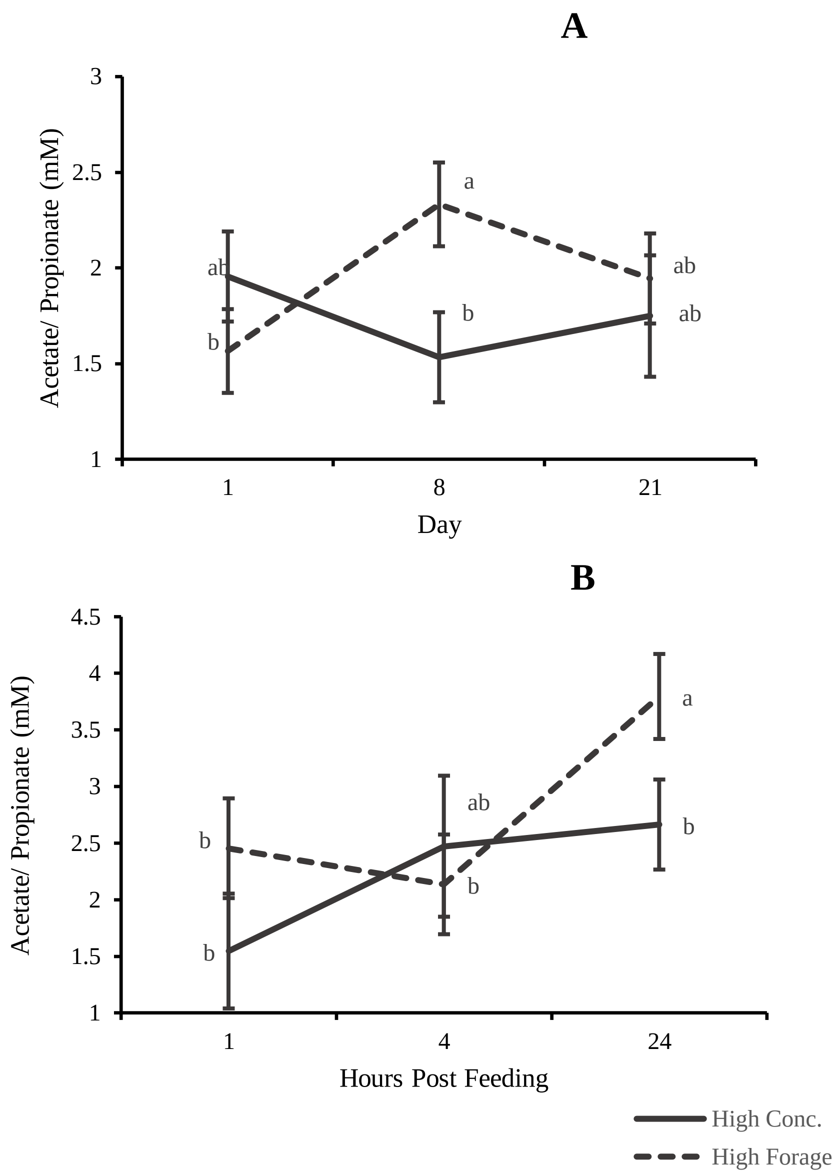
	Period						Diet			
	1	**2**	**3**	**4**	SEM	P-value	**High Conc.**	**High Forage**	SEM	P-value
Fermentation										
Total VFA	79.4 ^{c}	103.3 ^{a}	86.7 ^{bc}	104.3 ^{ab}	4.4	<0.01	98.6	86.9	3.1	0.01
Acetate (mM)	48.2	48.4	44.2	50.6	2.3	0.24	49.0	46.1	1.6	0.24
Propionate (mM)	18.7 ^{c}	38.3 ^{a}	32.4 ^{b}	39.3 ^{ab}	2.3	<0.01	36.0	30.4	1.6	0.01
Butyrate (mM)	14.5 ^{a}	16.6 ^{a}	10.1 ^{b}	14.4 ^{ab}	1.2	0.002	15.1	12.4	0.9	0.03
Acetate (mM %)	59.5 ^{a}	46.8 ^{c}	51.3 ^{b}	49.2 ^{b}	1.4	<0.01	49.6	52.4	1.0	0.03
Propionate (mM %)	22.8 ^{b}	37.2 ^{a}	37.4 ^{a}	37.3 ^{a}	1.2	<0.01	35.3	34.1	0.8	0.16
Butyrate (mM %)	17.7 ^{a}	16.0 ^{a}	11.3 ^{b}	13.4 ^{b}	0.8	<0.01	15.1	13.5	0.5	0.06
H_2 (%)	7.0 ^{a}	5.1 ^{ab}	2.9 ^{b}	3.1 ^{ab}	1.2	0.10	3.7	4.9	0.8	0.29
CH_4 (%)	17.0 ^{bc}	15.7 ^{c}	28.8 ^{a}	24.5 ^{ab}	2.6	<0.01	22.3	21.6	1.8	0.78
CO_2 (%)	75.9 ^{ab}	79.2 ^{a}	68.3 ^{b}	72.3 ^{ab}	2.5	0.01	74.1	73.5	1.8	0.82

$^{a–d}$ Means in the same row with different superscripts differ ($P < 0.05$).



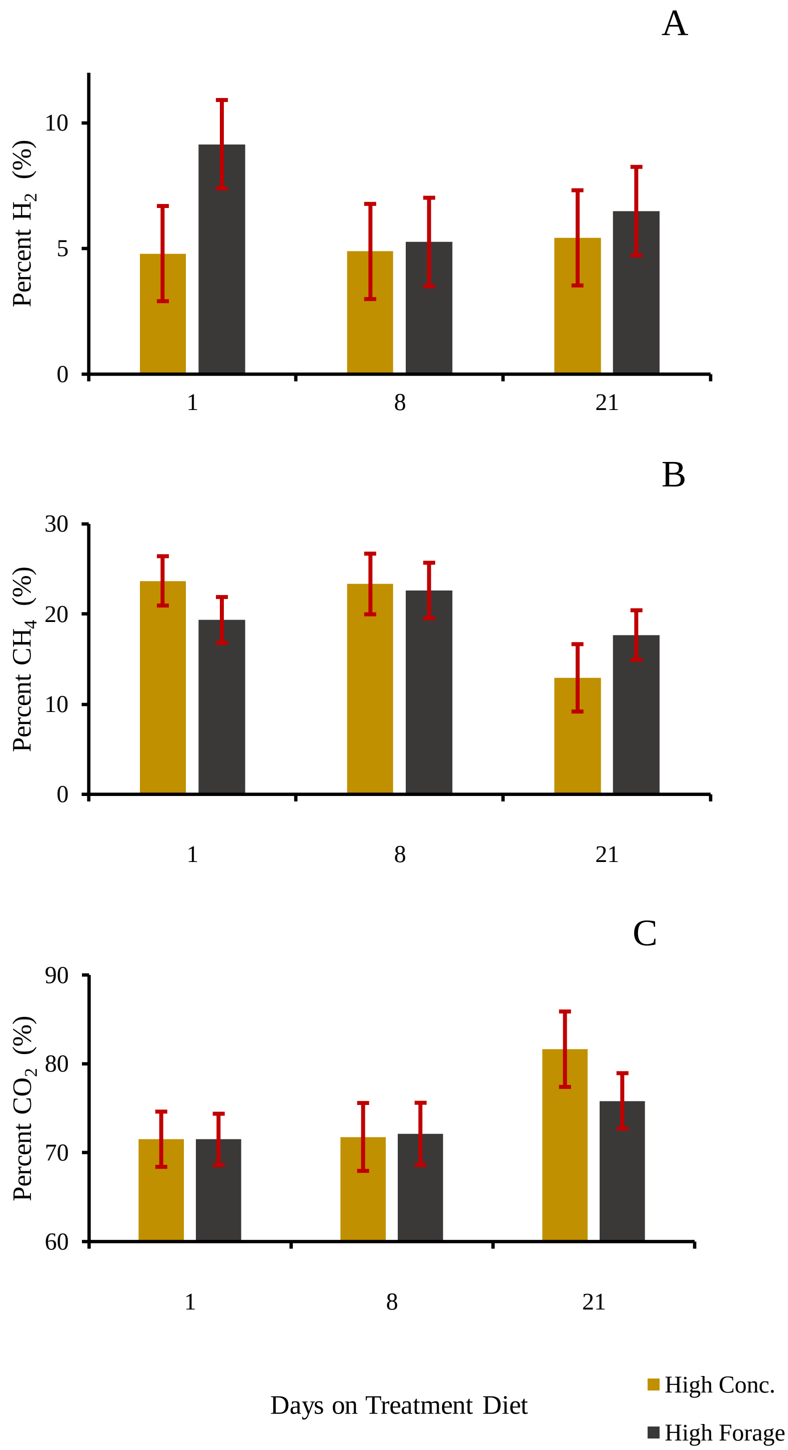
Each graph represents the overall changes in VFA concentration; (A) acetate, (B) propionate (C) butyrate between treatment diets on days 1, 8 and 21 of the feeding periods (n = 4)

Figure 3.2: Changes in VFA molar percentages between diets on days 1, 8 and 21 of feed experiment period.



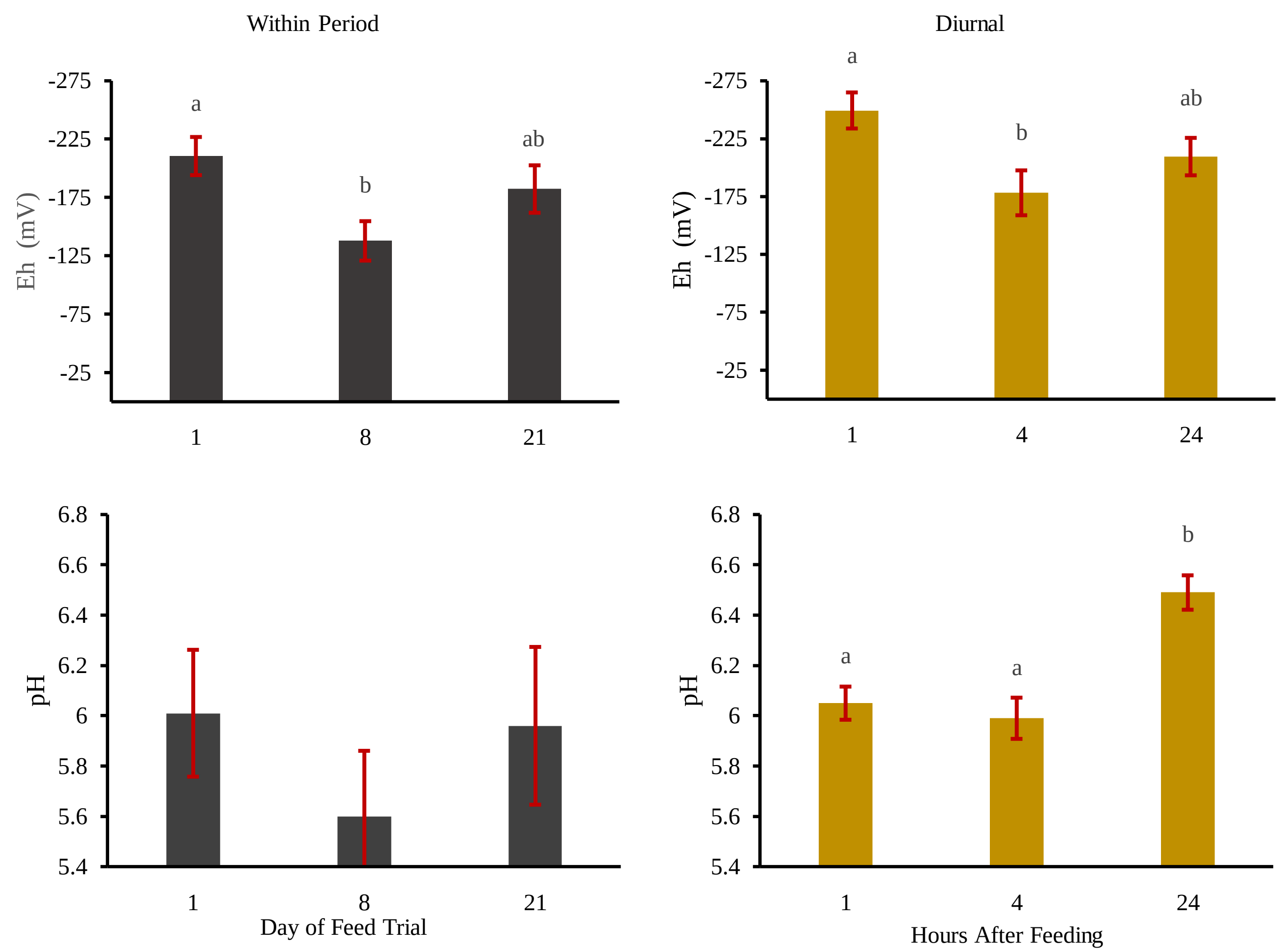
Within period graph (**A**) shows changes in rumen acetate to propionate ratio (A:P) on day 1, 8 and 21 of the feed experiment. Diurnal changes (right top and bottom) graphs changes A:P ratio 1, 4 and 24 hours after feeding on day 21 of the feed experiment

Figure 3.3: Diurnal and within-period differences in rumen pH and Eh.



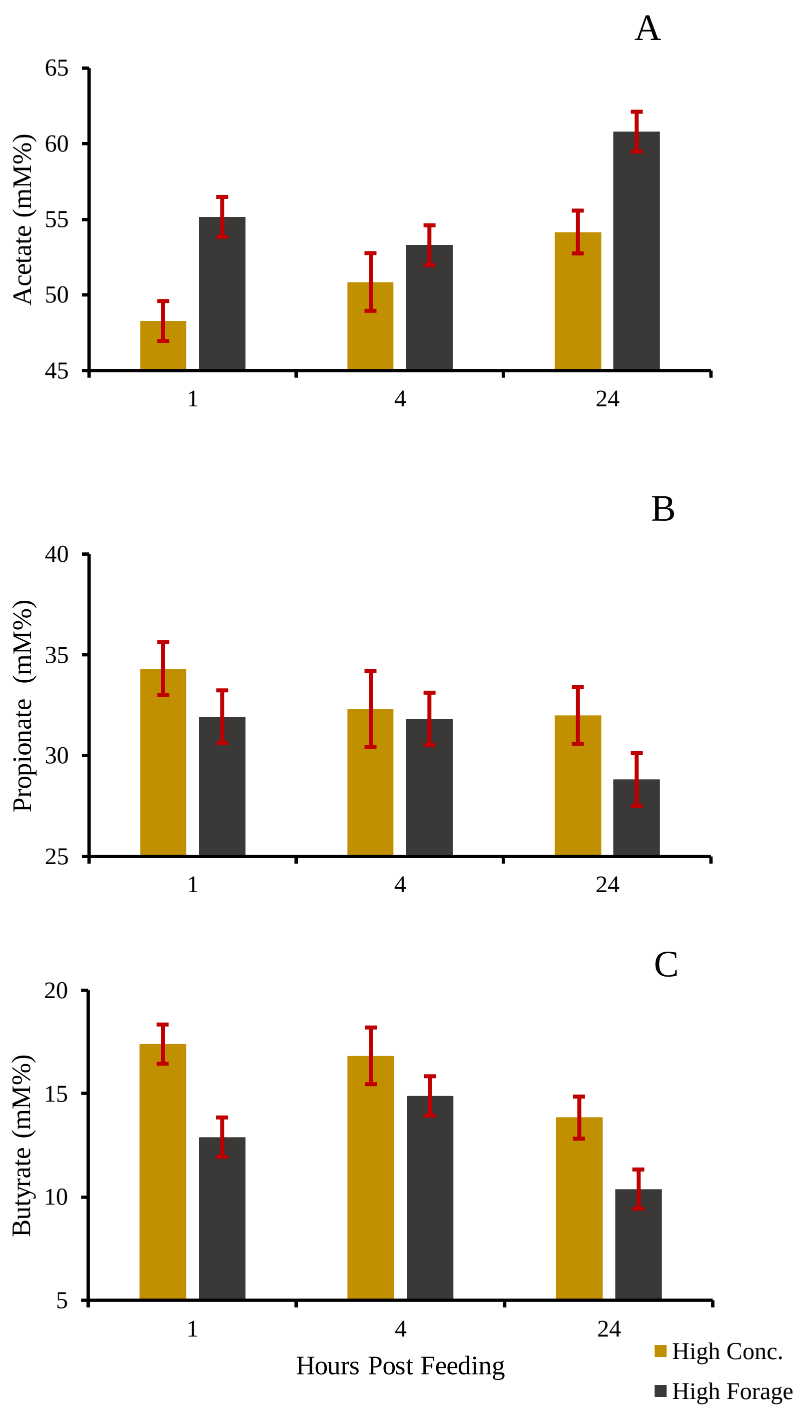
Each graph represents the overall changes in rumen gas percentage; (A) hydrogen, (B) methane (C) carbon dioxide between treatment diets on days 1, 8 and 21 of the feeding periods (n=4). Data represents percent of non-air fraction of 30 mL of gas collected directly from the rumen

Figure 3.4: Changes in rumen gas composition between diets on days 1, 8 and 21 of feed experiment.



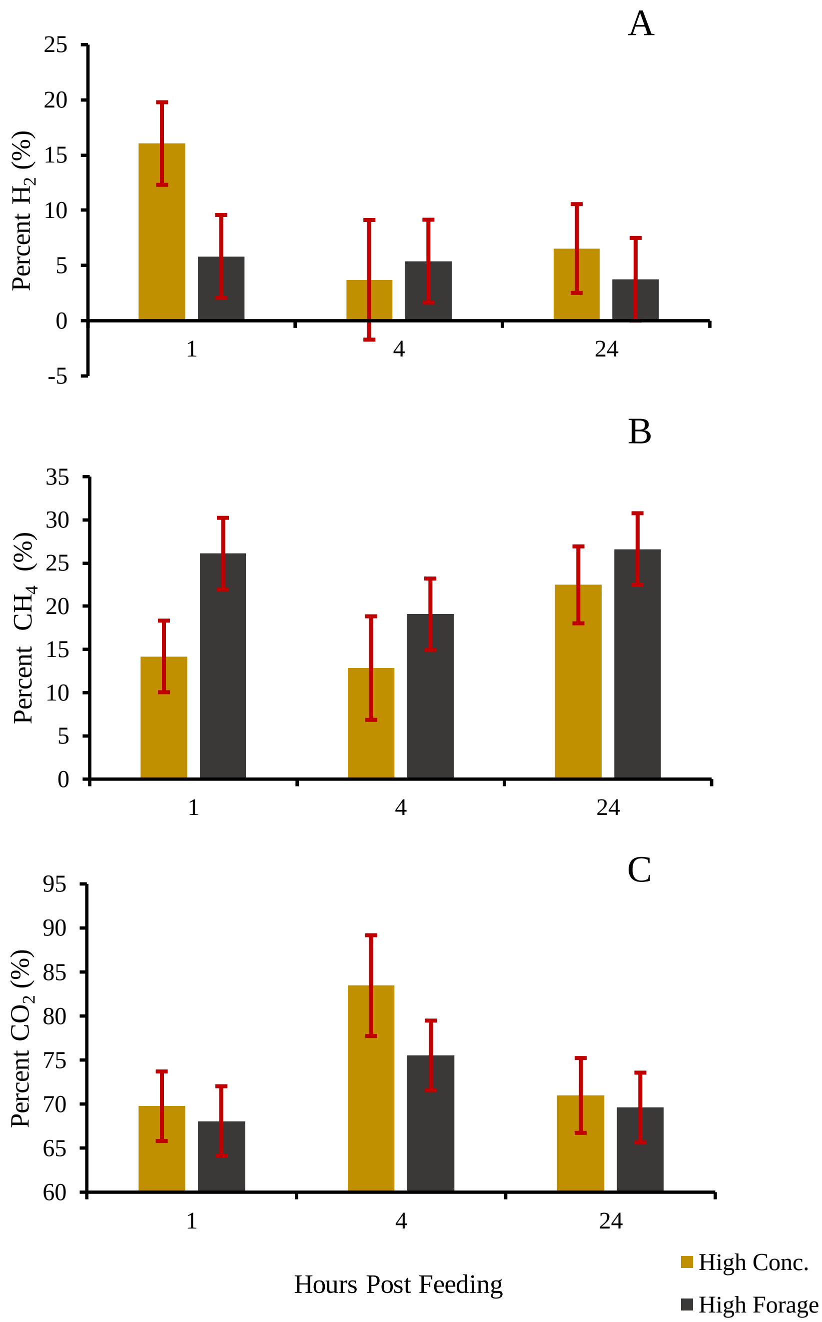
Within period graph (left top and bottom) shows changes in Eh and pH on day 1, 8 and 21 of the feed experiment. Diurnal changes (right top and bottom) graphs changes in Eh and pH 1, 4 and 24 hours after feeding on day 21 of the feed experiment.

Figure 3.5: Diurnal and within-period differences in rumen pH and Eh.



Each graph represents the overall changes in VFA molar percentages; (A) acetate, (B) propionate (C) butyrate between treatment diets 1, 4 and 24 hours after of the feeding on day 21 of the feeding experiment

Figure 3.6: Diurnal changes in VFA molar percentages 1, 4 and 24 hours after feeding**.**



Each graph represents the overall changes in fermentation gas percentage; (A) hydrogen, (B) methane (C) carbon dioxide between treatment diets 1, 4 and 24 hours after of the feeding on day 21 of the feeding experiment. Data represents percent of non-air fraction of 30 mL of gas collected directly from the rumen

Figure 3.7: Diurnal changes fermentation gas composition 1, 4 and 24 hours after feeding.

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Fermentation enzyme kinetics for rumen fluid from lactating cows on diets with different forage-to-concentrate ratios

ABSTRACT

Rumen fermentation process is controlled by thermodynamic and kinetic principles. Rates of fermentation depend on substrate and enzyme concentrations, and if products build up can also be limited by product concentrations. The objective of this study was to determine the enzyme activities, and substrate and product concentrations for rumen fermentation on different types of rations. Rumen fluid from four lactating dairy cows was used in a double switchback experimental design to test the effect of forage: concentrate ratio over four periods (2 diets by 4 periods) on 6 different fermentation intermediates analyzed by perturbation with 5 different levels of intermediates added *in vitro* in repeated measures. The treatment diets were formulated by replacing forage portions of the diet (triticale and corn silage) with concentrate portion (ground corn and SBM). This resulted in rations with forage-to-concentration ratios of 75:25 (HF) and 53:47 (HC) on a DM basis. Separate tubes of rumen fluid were incubated with 4 levels propionate, acetate, succinate, lactate, glucose or H_2. Na-acetate addition increased (P=0.0013) propionate mean concentrations for the two diets from 22.29 mM with no addition of acetate (0 mM) to 31.1, 32.1 and 34.1mM for 90-, 120- and 180-mM Na-acetate. Both Na-acetate and Na-propionate were successful in decreasing CH_4 percent in the final gas composition, CH_4 decreased from approximately 9% without any Na-acetate or Na-propionate to methane percentages ranging between 6.34-7.17%. Propionate rate of formation increased in rumen fluid incubated with increasing levels of Na-succinate (P=0.02), with rates ranging from 0.09, 1.70, 2.67 and 3.555 mM/hour respectively. Similar trends were observed for increasing propionate rate of formation for Na-lactate (P=0.02). Increasing the H_2 headspace % had no effect (P>0.05) on CH_4 volume or composition. Additionally, H_2 had no effect on the rate of formation of

propionate. Overall, addition of Na-acetate and Na-propionate was successful in inducing various shifts in in VFA profile while Na-succinate and Na-lactate highlights the ability prominence of both acrylate and succinate pathways of propionate production in cows adjusted to HC.

INTRODUCTION

Recent interest in strategies focused on improving sustainability and productivity of intensive animal production systems has given rise to research topics focused on greenhouse gas emissions from the dairy industry. Researchers have identified a wide variety of feed additives, supplements and management practices that have showed some success in reducing the greenhouse gas emission from ruminant species. Some of the most widely investigated strategies include altering forage and concentrate inclusion rates (Amat, McKinnon, et al., 2014; Wang, Zhang, et al., 2020), use of feed additives (Patra & Yu, 2014; Yang, Rooke, et al., 2016) or the use of microbial adaptations (Doyle, Mbandlwa, et al., 2019; Pereira, De Lurdes Nunes, et al., 2022) to name a few.

Successful strategies used to reduce enteric methane are suggested to increase the availability of dietary energy inputs by 3 – 10% of the gross energy (Soliva & Hess, 2007; Beauchemin, Ungerfeld, et al., 2020), however, very few studies have been successful in recovering or explaining the inability to effectively redirect the energy reserved from inhibiting methanogenesis (Emilio M. Ungerfeld, Aedo, et al., 2019). Under normal conditions metabolic hydrogen does not accumulate in the rumen, rather, it is transferred from rumen microbes to methanogens that use the hydrogen to reduce carbon dioxide through the hydrogenotrophic pathway. Additionally, studies have shown that diverting metabolic hydrogen to more valuable pathways such as the succinate or lactate (acrylate) where propionate can be synthesized. This repurposing of metabolic hydrogen can now be used to as a precursor to more important processes like gluconeogenesis while improving feed efficiency (Kruger Ben Shabat, Sasson, et al., 2016).
Altering the concentration of key metabolites serving as reactants and products in the synthesis of major VFA and fermentation gas can be used to understand the mechanisms involved in shifting

fermentation profiles when methane is inhibited. Thermodynamic principles coupled with altering metabolite concentration was previously used to investigate how increasing H_2 concentrations alters the dominant pathway for propionate synthesis (Janssen, 2010). Consequently, this study aimed to investigate how different diet regimes affect enzyme activities in rumen fluid. Perturbation studies that add various levels of fermentation intermediates can show when certain enzyme pathways are limited by enzyme capacity (e.g., Vmax). We hypothesized that incubating HF and HC rumen fluid with intermediates of the dominant VFA fermentation pathways will result in differences in enzyme activities, and substrate and product concentrations.

MATERIALS AND METHODS

All experimental procedures were approved by the University of Maryland Institutional Animal Care and Use Committee prior to the start of the feeding experiment (IACUC: Protocol number 1376980-2).

Animal Housing and Diet

Four multiparous Holstein Friesian cows (1565 ± 29.2 lbs.) were assigned to one of 2 diet treatments and housed in a freestall dairy barn fitted with individual calan gates. Prior to the start of the experiment experimental animal were fed from individually assigned calan boxes for a three-week feeding adjustment period. For the remainder of the study cows were fed two modified total mixed rations (TMR) formulated to represent the treatment diets and meet the NRC requirement for lactating dairy cows with daily feed adjusted to allow a 10% refusal. Feed and water were offered *ad libitum* and the experimental animals remained a part of the farm's regular milking schedule. An additional replacement cow was also housed with the experimental animals, bringing the total to five lactating cannulated cows. The replacement cow received the basal diet offered to the rest of the milking herd for the duration of the study, unless a replacement cow was needed.

The normal TMR offered to the milking herd was altered to create two different treatment diets for the experiment. Altering the concentrate (soybean meal and ground corn) and forage (triticale and corn silage) components of the basal diet yielded a high-forage (HF) and high-concentrate diet (HC) diet (Table 4.1). The forage-to-concentrate ratio was 75:25 and 53:47 (%DM) for the HF and HC diet respectively. Both treatment diets were isocaloric and nitrogenous while maintaining the recommended NDF, ADF, and NFC NRC requirements for lactating dairy cows (National Research Council, 2003).

Study Design

Two pairs of cows (n=4) were assigned to one of two treatment diets and used in a double switchback experimental design. Each pair of cows alternated between treatment diets for a total of 4 experimental periods with 6 different metabolites tested in each period. The experimental design for testing the effect of each (n=6) metabolite was 4 periods and 4 cows (n=16) with treatment diets nested within cow-period, and repeated measures within each treatment resulting in a total of 64 fermentation bottles (experimental units) per metabolite within cow-period nested in treatment diet. A 21-day diet adjustment period allowed each feeding pair (cows on similar treatment) to adjust to the opposing treatment diet for period-1 and facilitated a diet switch simulation on day-1 of the feeding experiment (Figure 4.1). The four feed experiment periods aligned with various physiological states in the lifecycle of a dairy cow that may be grouped in two distinct phases of metabolic demands. Periods 1 and 2 occurred during the antepartum and early lactation period respectively, while periods 4 and 5 both occurred during mid-lactation. Cows adjusted to their respective diets for 21 days before rumen fluid was collected from each cow to test the effects of fermentation intermediates. The week following the diet 21 day feeding experiment was reserved for a series of *in vitro* experiments, each *in vitro* was carried out 72 hours apart while animals remained on their respective experimental diets. Three pairs of *in vitro* experiments were conducted to test the effect of increasing levels of (*In Vitro*-I) sodium acetate (0, 90, 120 and 180 mM) and sodium propionate (0, 40, 60 and 100 mM); (*In Vitro*-II) sodium succinate (0, 15, 30 and 60 mM) and sodium lactate (0, 15, 25 and 50 mM) followed by (*In Vitro*-II) glucose (0, 10, 25 and 50 mM) (see Table 4.2).

Animal and Fermentation Bottle Preparation

Individual feed intake was recorded using the feed offered and feed refusal measurements from a data ranger feed mixer/scale. TMR was prepared fresh daily and samples of the TMR, ground corn, soybean meal, corn silage and triticale were collected on a weekly basis for composite TMR and individual ingredient nutrient analysis at the end of each period (wet chemistry analysis - Cumberland Valley Analytical Services, Waynesboro PA).

Timothy hay (0.3 g) was added to damp Wheaton bottles (124mL) to allow the hay to be fixed to the bottle during the gas perfusion process. Immediately before the headspace gas perfusion or making the bottles anerobic 10 mL of their respective treatment was added, each bottle received a butyl-stopper and secured in place with an aluminum crimped cap. Randomized bottles were perfused in groups of 8 by attaching each bottle to a 24-gauge needles used in an automated laboratory anerobic device. The automated anaerobic device was a mechanized vacuum pump attached to a CO_2 tank and designed to alternate between vacuuming and flushing bottles for a specified amount of time (5 minutes vacuum and 3-minute flush) over a known time period. The headspace gas in randomly selected Wheaton was analyzed prior to the study to confirm anerobic conditions and excess gas was removed to ensure that all fermentation started ≤ 1.1 but ≥ 1 atm of headspace gas. Nylon balloons were used to mix CO_2 and H_2 to achieve the desired H_2% for *In Vitro*-III. After each vacuum was applied, the gas mix in the respective nylon balloon was used to flush the H_2 Wheaton bottles. All Wheaton bottles were prepared and made anerobic no greater than 12 hours prior to the collection of rumen fluid for each *in vitro* experiment.

Rumen Fluid Collection and Processing

fluid was collected from rumen-fistulated lactating Holstein cows 4 hours after morning feeding and processed according to the guidelines outlined in Goering and Van Soest (1970). Both solid and liquid fractions of the rumen content were sampled anaerobically in 50 mL centrifuge tubes and then capped for transportation to the lab. Total rumen fluid sample was homogenized by approximately 20 seconds of pulsing in a blender made anaerobically using CO_2 perfusions. Homogenized rumen fluid was strained, through 4 layers of cheese cloth and a layer of glass wool, into a 1L flask. Rumen fluid was stirred continuously using a magnetic stir bar and heated gently while bubbling CO_2 to maintain rumen conditions until rumen fluid was distributed respective bottles. Thirty mL of processed rumen fluid was distributed to Wheaton bottles using a CO_2-flushed 60mL syringe fitted to a two valve and 23-gauge needle. Initial time 0 VFA samples were collected after 10 minutes of shaking at 39°C and stored at -20°C for later analysis. Rumen gas (time 0) was also collected and stored (maximum 24 hours storage in rubber syringes). Wheaton bottles returned to the incubator while shaking for 4 hours before 4-hour gas and VFA samples were collected.

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. Supernatant (0.7 mL) was removed and phosphoric acid (0.3 mL, 10% H3PO4) added (De-La Rubia et al., 2009). The gas chromatograph (Hewlett-Packard model 6890) with a 1.8 m length x ¼ in. outer diameter x 4 mm inner diameter packed GC column (60/80 Carbopack-11766 support, model 1-21641, 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 29 mL/min. The initial column temperature was 110°C held for 1 min, then increased to 180 °C (ramp of 10°C/min) for 1 min, then increased to 200 °C (ramp of 20°C/min) for 3 min, and a post-run temperature of 110°C. The detector temperature was 250°C with a hydrogen and air flow of 30 mL/min and 200 mL/min, respectively. VFA rate of formation was reported as mmol Hour $^{-1}$ and calculated using the following formula:

$$\text{VFA Rate of Formation (ROF)} = \frac{\text{D VFA concentration (mmol)}}{\text{D Time (h)}}$$

Headspace Gas Collection

Headspace gas was collected by connecting a 3-way Luer-lock stopcock valve to an empty 100mL Fortuna glass syringe. A 27-gauge needle was connected to the opposite end of the 3-way Luer stopcock, and an additional labeled plastic syringe fitted with a 2-way stopcock was attached to the third/perpendicular end of the 3-way stopcock. The post incubation headspace gas volume was measured by closing the 3-way stopcock towards the labeled plastic syringe and allowing excess gas to pass into the 100-mL glass syringe. After the total gas volume was recorded approximately 10 mL of gas was subsampled to the labeled plastic syringe for later analysis (within 24 hours).

Headspace Gas Analysis

Gas concentrations were measured using gas chromatography (Hewlett-Packard model 6890) with a 15.0 ft length × 1/8 in. outer diameter × 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). Argon was used as a carrier gas with a constant pressure at 35.0 psi, flow of 6.5 mL/min. The column remained isothermal 150°C and held for 9.5 min. Detector was set to 250°C with a reference flow of 56.0 mL/min and makeup gas (Helium) set to 7.0 mL/min. Individual gas rate of formation was reported as mmol Hour $^{-1}$ and calculated using the following formula:

$$\text{Gas Rate of Formation (ROF)} = \frac{\text{D Gas concentration (mmol)}}{\text{D Time (Hr)}}$$

Statistical Analysis

Statistical analyses were conducted using JMP Pro 15 (JMP®, Version 15.2.0 SAS Institute Inc., Cary, NC, 1989-2007). The model statement for was: $Y_{ijk} = m + P_i + D_j + L_k + (D \times L)_{ij} + e_{ijk}$ where P, D and L represented the fixed effects of period, treatment diet and metabolite level respectively. Cow was nested in diet and statistical differences were declared at 0.05 while a tendency toward statistical difference was declared at $P > 0.05$ but < 0.10.

RESULTS

Perturbation with Na-Acetate and Na-Propionate

Increasing *in vitro* starting acetate concentration increased (P=0.0013) propionate concentrations in rumen fluid from cows adjusted to both HF and HC diets (Table 4.2). Mean propionate concentrations between the two diets were significantly higher with the addition of Na-acetate with 90-, 120- and 180-mM Na-acetate averaging 31.1, 32.1 and 34.1mM compared to 22.29 mM without the addition of acetate. There was no observed dietary effect in total VFA, acetate, propionate or butyrate concentration, however, Na-acetate addition (90, 120 or 180 versus 0) resulted in numerically higher propionate concentration in the HC cows (Figure 4.2). Acetate-to-propionate (A:P) ratio was significantly greater (P=0.03) in the HF versus HC diet with no addition (0 mM Na-acetate) of metabolite, however there were no differences observed between treatments diets when Na-acetate was added (90, 120 and 190 mM) (Figure 4.3). The addition of Na-acetate decreased the volume in mL of total gas (P< 0.01), CH_4 (P< 0.01) and CO_2 (P< 0.01) compared with no addition. However, those results were only reproduced in the CH_4 gas composition (% of non-air fraction). Methane percent decreased (P= 0.04) from 9.4 % with 0 mM of Na-acetate to 6.61, 6.94 and 7.18 mM for 90, 120 and 180 mM of Na-acetate (Table 4.3). Increasing starting levels of sodium did not affect fermentation gas rate formation (ROF) (mM/hour) of H_2 (P= 0.47) or CH_4 (P= 0.14), however CO_2 ROF decreased from 0.51, 0.31, 0.31 and 0.19 mM/hour for 9, 90, 120 and 180 mM of Na-acetate, respectively.

The change in free energy for various fermentation reactions was calculated using calculated using the following equation:

$$\Delta G = \Delta G^{\circ} + RT \ln \{[\text{Products}] / [\text{Reactants}]\}$$

The following ΔG° were used to calculate ΔG and were derived using the assumptions of pH = 6.6[2], $[H^+] = 10^{-6.6}$, $[HCO_3]$ = 7 mol and $[H_2O]$ = 50 mol (E M Ungerfeld & Kohn, 2006; Van Lingen, Plugge, et al., 2016). Calculated ΔG for methanogenesis via CO_2 and H_2 reduction remained more negative in the HF compared to the HC diet, however ΔG were only significantly different without the addition of Na-acetate. Additionally, Na-acetate addition appears to lower the thermodynamic feasibility (became more less negative) of methanogenesis in the HF diet (Figure 4.4). The ΔG for methanogenesis in HF-adapted rumen fluid became less negative with increasing levels of Na-acetate with estimates of -97.89, -85.13, -89.56 and -87.99 for 0, 90, 120 and 180 mM respectively. Similar trends were observed in estimates for reductive acetogenesis (Figure 4.5) and the interconversion of acetate to propionate (Figure 4.5).

Increasing starting Na-propionate concentration *in vitro* decreased (P= 0.007) acetate concentrations from 69.11 to 64.67, 63.93 and 63.67 mM for 0, 40, 60 and 100 mM of Na-propionate, respectively. On average, rumen fluid adapted to HF diets also had greater (P= 0.02) concentrations of acetate (71.98 mM) compared to HC diets (66.23 mM). Acetate ROF declined significantly (P= 0.008) from 2.58 mM/hour when incubated with 0 mM of Na-propionate compared to 1.95, 1.12 and 1.42 mM/hour incubated with 40, 60 and 100 mM of Na-propionate, respectively (Table 4.4). Methane volume in the current study decreased (P= 0.01) from 19.85mL at 0 mM propionate addition to 11.9, 11.79 and 11.88 mL for 40, 60 and 100 mM of added f Na-propionate. Similar contrasts were observed for CO_2 volume (P= 0.02) and CO_2 composition (P= 0.04) of fermentation gases. There were differences observed in contrasting dietary influence in the response of H_2, CH_4 and CO_2 volume (P< 0.10) as well as CH_4 (P= 0.04) and H_2 (P= 0.03) (Table 4.4) after incubating with increasing levels of Na-propionate. Rumen fluid from cows adjusted to the HF diet was lower for all previously mentioned statistical differences except for H_2

% being lowest in the HC (0.94%) versus the HF (16%). Additionally, there were no effects (*P*>0.05) of diet or increasing levels of Na-succinate on the thermodynamic controls of methanogenesis, acetogenesis or acetate to propionate interconversion in the current study.

Perturbation with Na-Succinate and Na-Lactate

Incubating rumen fluid from cows on two different F:C ration with increasing levels of Na-succinate had no effect on the concentration (mM) of acetate (*P*= 0.17) or butyrate (*P*= 0.55), however there was a linear increase (*P*= 0.0001) in propionate concentration with 0, 15, 30 and 60 mM added succinate yielding 32.82, 40.27, 44.35 and 50.36 mM respectively. There was a dietary influence (*P*= 0.03) observed with greater propionate in the HC versus HF (39.37 versus 26.27 mM respectively). The results were supported by the differences observed in the rates of formation of VFA. Increasing succinate concentration tended to decrease (*P*= 0.09) acetate ROF while propionate ROF increased linearly (*P* < 0.01) (Table 4.5). HC treatment diet incubated with Na-succinate also increased the ROF of propionate (*P*= 0.03) and butyrate (*P*= 0.03) in addition to increasing the CO_2 volume (*P*= 0.02) and consequently total gas production (*P*= 0.07) in HC cows versus HF cows. The addition of succinate (15, 30 and 60 mM) decreased the methane ROF (*P*<0.01), and CH_4 % in headspace gas compared to no succinate addition (0mM Na-succinate). Addition there were no effects (*P*>0.05) of diet or increasing levels of Na-succinate on the thermodynamic controls of methanogenesis, acetogenesis or acetate to propionate interconversion in the current study.

Increasing lactate starting concentration has no significant effect on acetate, propionate, butyrate or total VFA mM concentration *in vitro*, however, there was a significant difference (*P*=0.002) in the A:P ration of samples incubated with additional lactate versus those without

(Table 4.6). Rates of formation estimates were highest for propionate (*P*= 0.02) and butyrate (*P*<0.01) only when incubated with the two highest levels of lactate (25 and 50 mM), similar trends were observed for the H_2 % of headspace gas (*P*= 0.04). Inversely, total gas and CO_2 volumes were highest (*P*< 0.05) only without the addition of Na-Lactate. Additionally, there were no effects (*P*>0.05) of diet or increasing levels of Na-lactate on the thermodynamic controls of methanogenesis, acetogenesis or acetate to propionate interconversion in the current study.

Perturbation with Glucose and Headspace H_2

Glucose addition in the current study tended to decrease (*P*=0.08) acetate concentration with 0, 10, 25 and 50 mM of added glucose yielding 78.3, 69.8, 70.9 and 72.9 mM of acetate respectively. There was no effect (*P*>0.05) of added glucose level or diet on propionate or butyrate concentrations. The apparent numeric decline in acetate rate of formation from 2.94 mM/hour when incubated with 0 glucose to 2.48, 2.10 and 1.71 mM/hour for 10, 25 and 50 mM of glucose was not statistically significant (*P*=0.18) (Table 4.7). Total gas volume decreased (*P*<0.01) from 216.33 mL without the addition of glucose to 193.42, 191.58 and 181.20 mL for 10, 25 and 50 mM of added glucoses. Rumen fluid from cows adapted to the HF diet had higher (*P*<0.05) A:P ratio (HC: 2.0 versus HF:2.69) and CH_4 volume (HC: 27.57 versus HF: 28.41mL) after 4 hours of incubation with increasing levels of glucose. Increasing glucose levels nor adapted diet had no effect on the rate of formation for individual VFAs or composition of headspace fermentation gas (Table 4.7). Additionally, there were no effects (*P*>0.05) of diet or increasing levels of glucose on the thermodynamic controls of methanogenesis, acetogenesis or acetate to propionate interconversion in the current study.

Incubating increasing headspace H_2 concentration with rumen fluid adapted to HC had higher acetate (HC: 94.13 versus HF: 90.16 mM, *P*=0.04), propionate (HC: 54.75 versus HF: 34.99 mM, *P*=0.005) and butyrate (HC: 31.45 versus HF: 21.41 mM, *P*=0.03) compared with cows adapted to HF diets. This resulted in higher (*P*<0.001) total VFA concentrations of 180.33 and 136.58 mM when contrasting HC and HF respectively. However, increasing headspace H_2 percent not different F:C ratio had no effect (*P*>0.10) on VFA ROF (Table 4.8).

DISCUSSION

Acetate inclusion inhibited acetate production and shifted fermentation away from acetogenesis and favored propionate production in the present study and resulted in lower ratio of A:P formation with Na-acetate addition. The decline in A:P formation ratio was also more noticeable in rumen fluid adjusted to HF diet. These findings were also observed in previous work that demonstrated addition of up to 50 mM of Na-acetate was successful in decreasing total fermentation gas production between 4 and 16 hours of incubation (Judd, 2018). Additionally, based on the theoretical principles explaining the influence of thermodynamics on rumen digestion, increasing levels of acetate would result in an increased product concentration and negatively affected the feasibility of acetogenesis via the glucose glycolysis to pyruvate pathway (R. a Kohn & Boston, 2000; E M Ungerfeld, 2006).

ΔG estimates for rumen fluid incubated *in vitro* with increasing levels of acetate demonstrates the fermentation shifts that occurred, with Na-acetate addition to HF rumen fluid shifting to more positive ΔG values for methanogenesis from CO_2 and H_2, and two possible alternative acetogenic pathways. The lower acetate rate of formation, CH_4 mL and CH_4 headspace composition support the effects observed changes in feasibility of acetogenic and methanogenic pathways. Lower methane in HF versus HC diets confirm the suggested thermodynamic shift in the HF rumen fluid methanogenesis.

Evidence of increasing levels of Na-propionate impeded the production of VFA was confirmed by the reduced concentration of propionate and acetate in the current study. Studies have shown the importance of the roles methanogens and other hydrogen sinks play in maintaining a healthy anaerobic fermentation ecosystem in the rumen, biodigesters or other fermentation chambers (Emilio M. Ungerfeld, 2015; P. He, Duan, et al., 2019). However, higher than usual propionate concentrations in the current study may have prevented the effective removal and thus accumulation of metabolic hydrogen. This was especially so in the HF diet-rumen fluid which was adapted to favoring acetogenesis accompanied by its H_2 byproduct. Therefore, high propionate concentration hindered effective removal of H_2 and H_2 accumulation resulted in lower acetogenesis in the HF diets. The conditions in the current experiment did not favor methanogenesis with H_2 accumulation.

Increasing propionate concentration was expected with increasing levels of Na-succinate or Na-lactate as succinate and lactate are fermentations intermediates that are readily converted to propionate in the rumen. Additionally, both Na-lactate and Na-succinate addition increased the rate of propionate formation linearly as expected. Furthermore, higher inclusion levels of succinate were successful in decreasing methanogenesis in the current study this methane inhibition may have occurred by the succinate propionate synthesizing pathway providing a hydrogen sink. Numerous studies have identified use of these propionate producing pathways as feasible strategies to alter propionate production in the rumen therefore improving VFA profile and reducing methanogenesis (Satter & Esdale, 1968; R. A. Kohn, 2015; Wang, 2020). Differences observed in propionate rate of formation between HF and HC diets support the understanding that rumen fluid from cows adjusted to HF diets may be using both the acrylate and succinate pathways simultaneously to produce propionate (Beauchemin, 2020; Pereira, 2022). The levels of glucose used in the present experiment were only successful in decreasing A:P ratio in HC diet and decreasing the total fermentation gas production. Increasing H_2 concentration resulted in higher propionate concentration as expected, however there was no effect of methane volume or headspace composition. The observed lack of response for methanogenesis contradicts earlier studies where increased H_2 availability increased CH_4 productions. However, similar observations with increased VFA production when headspace hydrogen increased from 0 to 36 and 60 mL of H_2. Although not expected, the increased acetate mM concentration was also observed in other studies in the literature but remain unexplained until further research.(Qiao, Tan, et al., 2015; Z. X. He, Qiao, et al., 2019).

CONCLUSION

The current study highlighted the shifts that occur in VFA and fermentation gas with the increasing levels of acetate and propionate. Additionally, the shifts and trends observed when rumen fluid adapted to HF or HC diet was incubated with different levels of succinate and lactate highlight the occurrence of propionate synthesis via both the acrylate and succinate pathway simultaneously, however there may be pathway variations in propionate rates of formation.

Table 4.1: Treatment Diet ingredients and formulation**.**

Feed Ingredients	**Diets** Basal[a] (%)	High-Forage (HF) Diet (%)	High-Concentrate (HC) Diet (%)
Corn Grain Ground[c]	8.43	3.21	17.45
Soybean Meal 48%[c]	4.51	4.83	7.27
Lactating Mineral	5.50	2.17	1.19
Megalac[b]	0.23	1.19	0.54
Triticale Silage[d]	25.81	36.91	29.89
Corn Silage[d]	55.52	51.69	43.66
F:C (as fed)	86:14	92:8	75:25
F:C (Dry Matter)	-	75:25	53:47
TOTAL	100.000	100.000	100.000

Each cow receives approx. 120 lbs. cow^{-1} day^{-1}

[a] Refers to the normal diet formulation of the normal diet offered to lactating cows at the Clarkesville Research Facility

[b] High-energy rumen-protected fat source included in diet

[c] Concentrate portion of the diet to be altered

[d] Forage portion of the diet to be altered

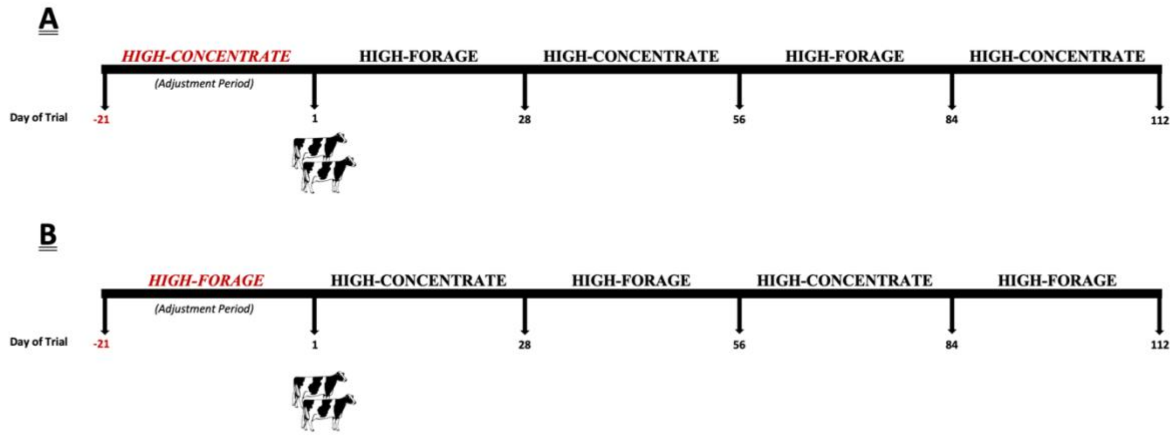

Figure 4.1: Feeding experiment timeline and treatment diet assignment to each pair of cows (A or B) in a four-period double switchback experimental design.

Table 4.2: Pairs of fermentation metabolite and treatment concentration tested *in vitro*.

		Level			
In- Vitro	Fermentation metabolite	**1** [c]	**2**	**3**	**4**
I	Sodium Acetate [a]	0	90	120	180
	Sodium Propionate [a]	0	40	60	100
II	Sodium Succinate [a]	0	15	30	60
	Sodium Lactate [a]	0	15	25	50
III	Glucose [a]	0	10	25	50
	$H_{2\ (gas)}$ [b]	0	15	30	60

[a] mM of metabolite in 10mL solution incubated with 30mL of rumen fluid
[b] Percent headspace gas mixed with CO_2 to flush Wheaton bottle headspace
[c] level one of liquid metabolites received 10 mL of *in vitro* media

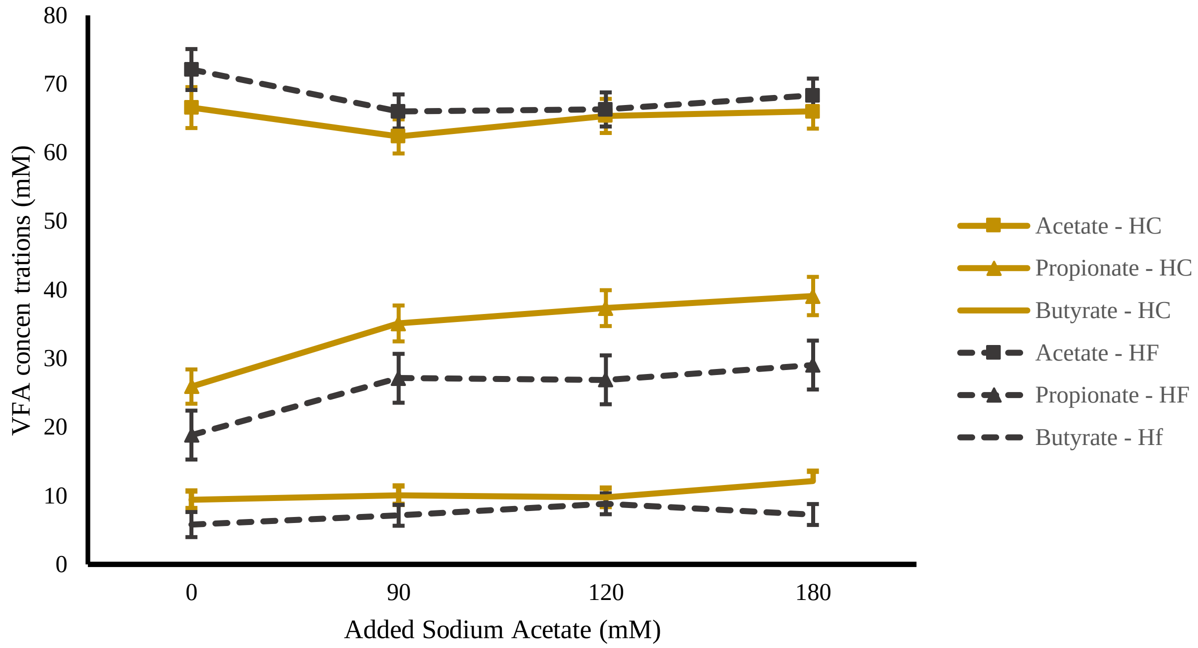

Figure 4.2: Effect of increasing acetate concentration and different F:C ratio on VFA concentration.

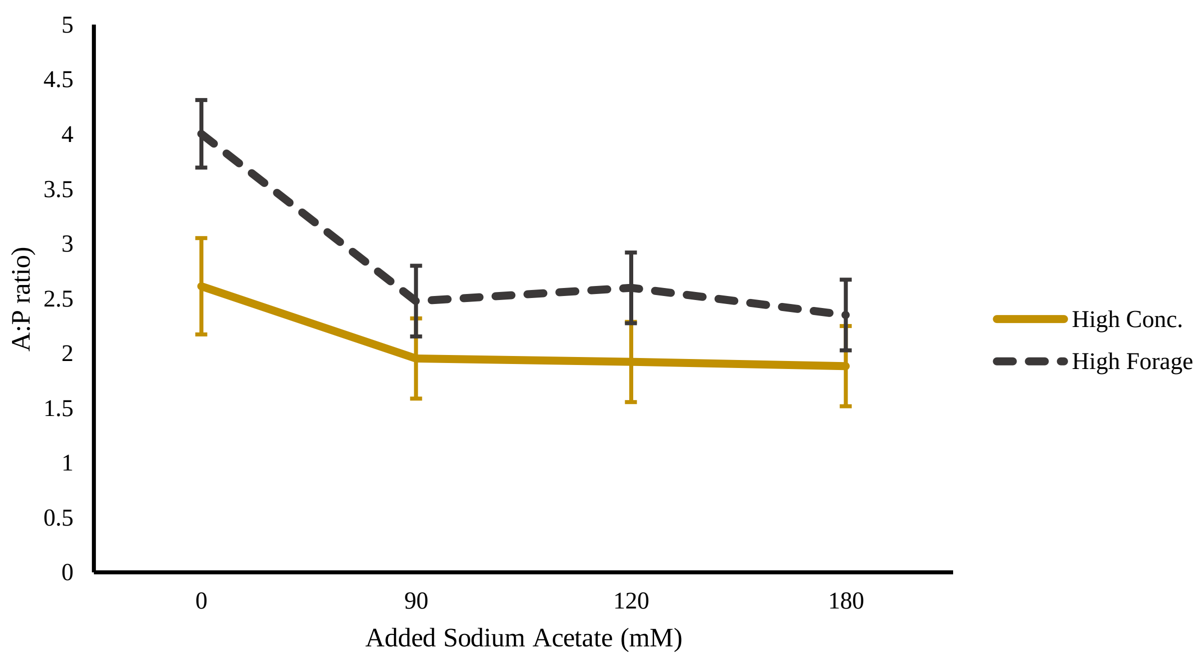

Figure 4.3: Effect of increasing acetate concentration and different F:C ratio on rumen A:P ratio.

Table 4.3: Effect of Na-Acetate perturbation and different F:C ratio of VFA and gas rates of formation**.**

	Na-Acetate Level (mM)								
	0	**90**	**120**	**180**	SEM	P-value	HC	HF	Diet
Total VFA	99.31	103.90	107.23	110.91	3.5	NS	101.9	96.7	NS
ROF (mM/ Hr)									
Acetate	2.04	1.37	1.79	1.89	0.36	NS	1.9	2.19	NS
Propionate	-0.005	0.83	1.26	1.13	0.37	NS	-0.07	0.06	NS
Butyrate	0.92	0.85	0.98	1.09	0.16	NS	1.08	0.76	NS
Total Gas (mL)	211.38 ^{a}	184.41 ^{b}	189.01 ^{b}	180.89 ^{b}	5.07	<0.01	101.87	96.74	NS
H_2	0.68	0.79	0.95	7.54	3.1	NS	1.04	0.32	NS
CH_4	19.98 ^{a}	12.25 ^{b}	13.21 ^{b}	13.35 ^{b}	1.67	<0.01	22.42	17.53	NS
CO_2	190.73 ^{a}	171.38 ^{b}	174.85 ^{ab}	160.00 ^{b}	5.52	<0.01	194.4	187.04	NS
Gas (%)									
H_2	0.29	0.42	0.48	3.89	0.03	NS	0.49	0.08	NS
CH_4	9.40 ^{a}	6.61 ^{b}	6.94 ^{b}	7.18 ^{b}	0.72	0.04	10.11	8.71	NS
CO_2	90.31	92.97	92.58	88.92	1.69	NS	89.41	91.21	NS

$^{a–d}$ Means in the same row with different superscripts differ ($P < 0.05$).

* Gas percent calculated as a fraction of total non-air

Table 4.4: Effect of Na-propionate perturbation and different F:C ratio of VFA and gas rates of formation.

	Na-Propionate Level (mM)								
	0	**40**	**60**	**100**	SEM	P-value	HC	HF	Diet
Total VFA	103.13	100.73	100.58	101.80	2.56	NS	103.72	102.55	NS
ROF (mM/ Hr)									
Acetate	2.58 [a]	1.95 [ab]	1.12 [b]	1.42 [b]	0.31	0.008	1.96	1.53	NS
Propionate	0.35	0.82	0.49	0.17	0.41	NS	0.79	0.08	NS
Butyrate	1.09	0.77	0.54	0.76	0.16	NS	0.98	0.58	NS
Total Gas (mL)	103.13	100.73	100.58	101.80	2.56	NS	103.70	102.50	NS
H_2	8.48	0.90	0.99	1.51	3.30	NS	0.94	16.02	0.03
CH_4	19.85 [a]	11.90 [b]	11.79 [b]	11.88 [b]	1.98	0.01	16.89	10.93	0.002
CO_2	189.20 [a]	167.23 [ab]	179.65 [b]	169.60 [b]	5.39	0.02	198.98	179.42	0.08
Gas (%)									
H_2	8.48	0.90	0.99	1.51	3.30	NS	0.40	7.30	0.03
CH_4	9.00	6.64	6.34	6.57	0.89	NS	11.65	6.34	0.004
CO_2	87.13 [a]	92.90 [b]	93.17 [b]	92.65 [b]	1.72	0.05	87.93	86.33	NS

[a–d] Means in the same row with different superscripts differ ($P < 0.05$).

* Gas percent calculated as a fraction of total non-air

Table 4.5: Effect of Na-succinate perturbation and different F:C ratio of VFA and gas rates of formation.

	Na-Succinate Level (mM)								
	0	**15**	**30**	**60**	SEM	P-value	HC	HF	Diet
Total VFA	107.67	109.23	111.27	119.29	7.06	NS	117.08	95.66	0.05
A:P ratio	2.32 [a]	1.60 [b]	1.38 [b]	1.26 [b]	0.18	< 0.01	1.812.61	2.75	0.01
Rate of Formation (mM/ Hr)									
Acetate	2.46 [a]	1.74 [ab]	1.25 [b]	1.51 [ab]	0.35	0.09	2.88	2.04	NS
Propionate	0.09 [c]	1.70 [b]	2.67 [ab]	3.55 [a]	0.48	<0.001	1.11	-0.93	0.03
Butyrate	0.65	0.74	0.64	0.61	0.13	NS	0.96	0.35	0.03
Total Gas (mL)	204.92	190.24	193.44	189.63	5.51	NS	215.21	194.63	0.07
H_2	0.38 [a]	0.34 [b]	0.38 [a]	0.36 [ab]	0.01	0.05	0.39	0.36	NS
CH_4	35.29 [a]	17.03 [b]	16.90 [b]	19.26 [b]	2.89	<0.01	33.02	37.57	NS
CO_2	169.25	172.87	176.16	170.01	6.31	NS	181.80	156.69	0.02
Gas (%)									
H_2	0.20	0.17	0.19	0.20	0.01	NS	0.19	0.21	NS
CH_4	16.67 [a]	9.18 [b]	8.88 [b]	9.92 [b]	2.42	0.07	15.15	18.19	NS
CO_2	83.13 [b]	90.65 [a]	90.92 [a]	89.88 [a]	1.28	0.07	84.66	81.60	NS

[a–d] Means in the same row with different superscripts differ ($P < 0.05$).
* Gas percent calculated as a fraction of total non-air

Table 4.6: Effect of Na-Lactate perturbation and different F:C ratio of VFA and gas rates of formation.

	Na-Lactate Level (mM)								
	0	**15**	**25**	**50**	SEM	P-value	HC	HF	Diet
Total VFA	104.10	104.61	110.30	114.93	5.74	NS	112.27	95.94	NS
A:P ratio	2.39 [a]	1.83 [b]	1.64 [b]	1.55 [b]	0.15	0.002	2.26	2.51	NS
Rate of Formation (mM/ Hr)									
Acetate	2.20	0.95	1.26	1.31	0.30	0.05	2.40	1.99	NS
Propionate	-0.57 [b]	0.73 [ab]	1.31 [a]	1.61 [a]	0.46	0.02	0.27	-1.43	0.09
Butyrate	0.43 [c]	0.57 [bc]	0.93 [ab]	1.33 [a]	0.16	< 0.01	0.72	0.13	NS
Total Gas (mL)	214.23 [a]	175.83 [b]	174.24 b	170.26 [b]	5.80	0.01	219.35	210.11	NS
H_2	0.37	0.34	0.35	0.41	0.03	NS	0.38	0.36	NS
CH_4	29.82	18.45	20.18	20.62	3.66	NS	31.14	28.49	NS
CO_2	184.04 [a]	157.05 [b]	153.71 [b]	149.23 [b]	6.29	<0.01	186.82	181.25	NS
Gas (%)									
H_2	0.17 [b]	0.20 [b]	0.22 [ab]	0.28 [a]	0.03	0.04	0.17	0.18	NS
CH_4	13.99	10.27	11.34	11.25	1.82	NS	14.62	13.36	NS
CO_2	85.84	89.53	88.44	88.47	0.73	NS	85.21	86.46	NS

[a–d] Means in the same row with different superscripts differ ($P < 0.05$).
* Gas percent calculated as a fraction of total non-air

Table 4.7: Effect of Glucose perturbation and different F:C ratio of VFA and gas rates of formation.

	Glucose Level (mM)								
	0	**10**	**25**	**50**	SEM	P-value	HC	HF	Diet
Total VFA	135.13	127.36	129.91	139.66	6.81	NS	142.17	128.09	NS
A:P ratio	2.35	2.01	2.00	1.82	0.18	NS	2.00	2.69	0.08
Rate of Formation (mM/ Hr)									
Acetate	2.94	2.48	2.10	1.71	0.39	NS	3.19	2.69	NS
Propionate	0.77	1.22	1.13	1.77	0.48	NS	1.14	0.40	NS
Butyrate	0.37	0.59	0.35	1.26	0.31	NS	-0.33	0.38	NS
Total Gas (mL)	216.33 [a]	193.42 [b]	191.58 [b]	181.20 [b]	5.18	< 0.01	210.00	222.67	NS
H_2	0.55	0.73	0.87	0.61	0.25	NS	0.57	0.54	NS
CH_4	27.99	19.06	20.50	17.22	4.30	NS	27.57	28.41	< 0.01
CO_2	187.79	173.62	170.21	163.37	5.23	NS	181.86	193.71	NS
Gas (%)									
H_2	0.25	0.37	0.46	0.33	0.13	NS	0.28	0.23	NS
CH_4	12.94	10.31	10.97	9.93	2.25	NS	12.60	13.28	NS
CO_2	86.81	89.32	88.57	89.75	2.22	NS	87.12	86.49	NS

[a–d] Means in the same row with different superscripts differ ($P < 0.05$).
* Gas percent calculated as a fraction of total non-air

Table 4.8: Effect of headspace H_2 perturbation and different F:C ratio of VFA and gas rates of formation.

	Headspace H_2 (%)								
	5	**15**	**30**	**60**	SEM	P-value	HC	HF	Diet
Total VFA	158.46	174.36	162.23	155.15	6.81	NS	180.33	136.58	< 0.01
A:P ratio	2.06	2.00	2.05	2.13	0.17	NS	1.76	2.35	0.08
Rate of Formation (mM/ Hr)									
Acetate	2.81	4.77	2.76	1.95	0.88	NS	3.93	1.69	NS
Propionate	2.15	3.53	2.20	1.32	0.73	NS	3.38	0.92	NS
Butyrate	3.00	4.63	3.61	2.57	1.06	NS	4.22	1.78	NS
Total Gas (mL)	178.23	189.30	178.36	174.94	5.65	NS	183.86	172.60	NS
H_2	1.85 ^{b}	7.00 ^{b}	12.03 ^{b}	27.37 ^{a}	3.94	< 0.01	1.79	1.92	NS
CH_4	26.05	32.34	28.62	38.75	5.62	NS	31.21	20.89	NS
CO_2	150.32 ^{a}	149.96 ^{a}	137.71 ^{a}	108.82 ^{b}	9.19	< 0.01	150.85	149.79	NS
Gas (%)									
H_2	1.04 ^{b}	3.70 ^{b}	6.63 ^{b}	16.28 ^{a}	2.41	< 0.01	0.96	1.12	NS
CH_4	14.64	16.93	16.13	22.19	3.16	NS	16.97	12.31	NS
CO_2	84.32 ^{a}	79.37 ^{a}	77.24 ^{a}	61.52 ^{b}	4.17	< 0.01	82.06	86.57	NS

$^{a–d}$ Means in the same row with different superscripts differ ($P < 0.05$).
* Gas percent calculated as a fraction of total non-air

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