ABSTRACT

Title dissertation: EFFECTS OF SUPPLEMENTING METABOLITES ON RUMEN FERMENTATION AND MILK PRODUCTION IN DAIRY COWS

Anand Tiwari, Doctor of Philosophy, 2026

Dissertation directed by: Professor Dr. Richard A. Kohn
Department of Animal and Avian Sciences

Microbial fermentation provides essential precursors necessary for milk formation in dairy animals. Archaeal methanogens use some of the available energy to form methane gas. Several nutritional additives have been suggested to change the pattern of ruminal fermentation in ways that could decrease methane emissions or improve efficiency. For example, red algae can directly inhibit methanogens, probiotics may shift fermentation to certain pathways, and metabolites like sulfite and acetate may change metabolic pathways. This study investigated the influence of the combination of such factors on rumen fermentation and milk production and feed intake of dairy cattle.

Chapter 1 reviews the literature associated with rumen fermentation to produce volatile fatty acid precursors for metabolizale energy of ruminants. Concepts such as carbohydrate fermentation, thermodynamic and kinetic effects on the profile of

metabolites, the various hydrogen sinks and sources, how hydrogen is partitioned, and effects and mechanisms of feed additives are reviewed.

In Chapter 2, an in vitro batch culture system with timothy hay substrate was used to test the effects of *Lactobacillus plantarum* and red algae (*Asparogopsis taxiformis*), alone or combined, on gas production dynamics. Red algae consistently reduced methane fraction and cumulative methane production from 0 to 72 h, while increasing hydrogen accumulation and initially suppressing total gas production before it exceeded the control at later incubation times; carbon dioxide parameters were unaffected, indicating strong anti-methanogenic potential that warrants further study of hydrogen redirection in alternative sinks.

In Chapter 3, an in vitro dose-response experiment investigated four levels of sodium sulfite (0 - 0.4 mM) on fermentation of timothy hay. The highest sulfite concentration reduced total gas production and methane at early time points (4 - 8 h), increased net hydrogen production at 4 h, and temporarily depressed volatile fatty acid (VFA) concentrations, however, fermentation recovered by 24 h, suggesting delayed but not permanently affecting fermentation in vitro.

In Chapter 4, 32 multiparous Holstein cows in a replicated 4 × 4 Latin square received diets containing three levels of sodium sulfite (0, 0.45, 0.9% of DM) with or without 1% sodium acetate to evaluate effects on intake, body weight, and milk production and composition. The treatments were mixed up in the corn based total mixed ration (TMR). Cows fed 0.45% sulfite had the greatest body weight, whereas 0.9% sulfite reduced milk yield and both sulfite levels lowered dry matter intake compared with the control. Sodium acetate did not affect milk yield, energy-corrected milk, or milk

composition, and neither additive altered fat, protein, or fatty acid percentages, contrary to the original hypothesis that sulfite would improve milk yield via increased propionate and that acetate would enhance milk fat.

Chapter 5 focused on rumen gas composition, VFA, pH, and redox potential (Eh) in cannulated cows using an unreplicated 4 × 4 Latin square design. The cows were fed one of four diets: bicarbonate, bicarbonate + sulfite, acetate, or acetate + sulfite. Ruminal samples were collected on Days 0, 3, 7, 14, 17, and 20 of each period, with additional samples collected on Day 17 at 0, 4, and 8 hours after feeding. Sodium sulfite and acetate, alone or in combination, did not affect mean rumen H_2, CH_4, CO_2 fractions, CH_4/CO_2 ratio, VFA concentrations, acetate:propionate ratio, or diurnal patterns of fermentation variables, whereas substantial variation was associated with period and sampling time, including lower pH and less negative Eh at 8 h post-feeding ($p<0.05$). This study suggested that the sulfite dose used in this trial did not affect the anti-methanogenic or VFA shifts in lactating cows. Therefore, further investigation was needed to explore the adaptability of ruminal micro-organism under such dietary conditions.

Finally, Chapter 6 used rumen fluid from cows previously exposed to dietary sulfite as donor inoculum for a 24-h in vitro batch culture to evaluate microbial adaptation. Donor diet had no effects on total gas production and VFA profiles, but in vitro sulfite addition again reduced gas and methane production, increased hydrogen accumulation, and tended to decrease acetate concentration and the acetate:propionate ratio. These findings indicate that, even after several weeks of exposure to dietary sulfite,

the rumen microbiota did not fully adapt to eliminate the anti-methanogenic response, as sulfite infusion in vitro changed gas dynamics and VFA patterns.

Collectively, these studies show that red algae and sulfite effectively suppressed methane and altered hydrogen dynamics in vitro. In contrast, under the conditions of the in vivo study, sulfite inclusion did not produce clear or consistent effects on rumen fermentation, rumen gas profiles, or performance in high-producing dairy cows. The limited replication and challenges associated with high-air rumen gas samples may have reduced the ability to detect treatment differences. Therefore, further work is needed to optimize dose, sampling methodology, and co-substrates to better evaluate the practical potential of sulfite for methane mitigation.

EFFECTS OF SUPPLEMENTING METABOLITES ON RUMEN FERMENTATION AND MILK PRODUCTION IN DAIRY COWS

By

Anand Tiwari

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Advisory Committee:

Professor Dr. Richard A. Kohn, Chair/Advisor

Professor Dr. Stephanie Lansing, Deans Representative

Associate Professor Dr. Nishanth E. Sunny

Assistant Professor Dr. Fabiana De Freitas Cardoso

Assistant Professor Dr. Eduardo Rico

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TABLE OF CONTENTS

ACKNOWLEDGEMENTS ..... **ii**

TABLE OF CONTENTS ..... **iv**

LIST OF TABLES ..... **vii**

LIST OF FIGURES ..... **ix**

Chapter 1: INTRODUCTION **1**

Literature review **4**

Carbohydrate metabolism and rumen environment 4

Thermodynamics and kinetics in ruminal fermentation: 5

Fatty acid production and utilization 7

Production and fate of metabolic hydrogen in the rumen 8

Alternative hydrogen sink 12

Feed additives 15

Chapter 2: Evaluation of the combined effect of red algae (*Asparogopsis taxiformis)* and probiotics (*Lactobacillus planturum*) on fermentation parameters in vitro **23**

ABSTRACT **24**

INTRODUCTION **26**

MATERIALS AND METHODS **28**

Rumen fluid collection and in vitro preparation 28

Gas and VFA samplings 29

Gas equation 30

Statistical analysis 31

RESULTS **32**

Total gas and cumulative gas 32

Hydrogen 32

Methane 33

DISCUSSION **33**

Gas parameters 33

Summary 36

Chapter 3: Effect of sodium sulfite on gas and volatile fatty acids parameters in vitro **41**

ABSTRACT **42**

INTRODUCTION **44**

MATERIALS AND METHODS **46**

Rumen fluid collection and preparation 46

Treatment 47
Gas Sampling 47
Gas analysis on GC 47
Gas equations 48
Statistical analysis 49
RESULTS 50
Total gas 50
Hydrogen 50
Methane 51
Carbon dioxide 51
Volatile Fatty Acid Parameters 51
DISCUSSION 52
Gas production 52
Volatile Fatty Acids 55
A:P ratio and Total VFA 57
Summary 58
Chapter 4: Effect of sodium sulfite or sodium acetate on feed intake and milk production in dairy cattle 64
INTRODUCTION 67
MATERIALS AND METHOD 68
Animal, housing and feeding 68
Feed intake, body weight and body condition scores 70
Silage sampling and DM intake 70
Milk samples 71
Statistical Method 71
RESULTS 73
Milk yield 74
Milk composition and fatty acid profile 75
Period effect 76
DISCUSSION: 77
DM intake: 77
Body weight and BCS 80
Milk production and Milk composition 82
Milk components 84
Period effect 85
Summary 88
Chapter 5: Effect of sodium sulfite or sodium acetate on ruminal gas and VFA parameters 98

ABSTRACT **99**

INTRODUCTION **101**

MATERIALS AND METHODS **103**
Animal, housing and feeding 103
Rumen Sampling 104
Gas sampling 104
pH and Eh 105
Data analysis 105

RESULTS **107**
Gas and Volatile fatty acid 107
pH and Eh 107

DISCUSSION **108**
Gas and Volatile Fatty Acid 108
pH and Eh 111
Summary 112

Chapter 6: Does adaptation to sodium sulfite in the rumen attenuate its effect on methane emissions **120**

ABSTRACT **121**

INTRODUCTION **124**

MATERIALS AND METHOD **126**
Animal, housing and feeding 126
Rumen fluid collection 126
Gas and fluid sample collection 127
Gas equation 128
Statistical analysis 129

RESULTS **130**
Effects of donor diets 130
Effects of in vitro treatment 130
Donor treatment x In vitro sulfite 132

DISCUSSION **133**
Gas parameters 133
Volatile fatty acids 136
Summary 137

LIST OF TABLES

Table 2.1: The effect of Asparogopsis taxiformis, Lactobacillus plantarum, and their combination on gas parameter at different time 0, 4, 24, 48 and 72 hours in vitro. 37

Table 2.2: The effect of Asparogopsis taxiformis and Lactobacillus plantarum on H_2, CH_4 and CO_2 concentration at different time interval 0, 4, 24, 48 and 72 hours in vitro. 38

Table 2.3: The effect of Asparogopsis taxiformis and Lactobacillus plantarum on net H_2, CH_4 and CO_2 production at different time interval 0, 4, 24, 48 and 72 hours in vitro. 39

Table 2.4: The effect of Asparogopsis taxiformis, Lactobacillus plantarum and their combination on cumulative H_2, CH_4 and CO_2 production at different time interval 0, 4, 24, 48 and 72 hours in vitro. 40

Table 3.1: The effect of different levels of sodium sulfite on total gas (mL) and cumulative gas (mL) production at 0, 4, 8, 24, and 48 hours of time interval in vitro..... .59

Table 3.2:The effect of different levels of sodium sulfite on concentration of H_2, CH_4 and CO_2 at 0, 4, 8, 24, and 48 hours of time interval……..60

Table 3.3: The effect of different levels of sodium sulfite on net H_2, CH_4 and CO_2 at 0, 4, 8, 24, and 48 hours of time interval. 61

Table 3.4: The effect of different levels of sodium sulfite on cumulative gas production at 0, 4, 8, 24, and 48 hours of time interval. 62

Table 3.5: Least square mean concentration of acetate, propionate, butyrate, total VFA and A:P ratio at different levels of sodium sulfite inclusion across 0, 4, 8, 24, and 48-hour time points 63

Table 4.1: Ingredient and nutrient composition of the lactating cow diet 89

Table 4.2: Effects of sodium sulfite and sodium acetate on body weight, body condition score, feed intake (as-fed basis), dry matter intake, milk yield, and feed efficiency (milk yield/DMI) in lactating dairy cows, adjusted for baseline milk yield and days in milk..... 91

Table 4.3: Effects of dietary sulfite level x week on milk yield and dry matter intake of dairy cows. 92
Table 4.4: Effects of dietary sodium acetate level x week on milk yield and dry matter intake of dairy cows. 93
Table 4.5: Effect of experimental period on body weight, BCS, feed intake (As fed kg), DMI, Milk yield (kg/day), and Milk/DMI (kg/kg) in lactating dairy cattle. 94
Table 4.6: Effect of sodium sulfite and sodium acetate supplementation on milk composition and fatty acid profile in lactating dairy cows. 95
Table 4.7: Effect of period on milk composition, fatty acid composition, and individual fatty acid profile in lactating cow. 96
Table 4.8: Fixed effects tests for Milk yield kg/day, Dry matter intake and feed efficiency..... 97

Table 5.1: Allocation of 4 treatment in 4 groups of cannulated cows across four period in 1 X 1 Latin Square design..... 114
Table 5.2: Ingredient and nutrient composition of the lactating cow diet 114
Table 5.3: Effect of Bicarbonate, Bicarbonate + Sulfite, Acetate and Acetate + Sulfite on individual ruminal gas fraction and CH_4/CO_2 in dairy lactating cows. 116
Table 5.4: Effect of period on individual ruminal gas fraction and CH_4/CO_2 in dairy lactating cows..... 116
Table 5.5: Effect of Bicarb, Bicarb + Sulfite, Acetate and Acetate + Sulfite on pH, Eh, individual ruminal VFA, total VFA, and A:P in dairy lactating cows. 117
Table 5.6: Effect period on individual ruminal pH, Eh, acetate, propionate, butyrate, A:P and total VFA in dairy lactating cows. 117
Table 6.1: The effect of donor diet on total gas and cumulative gas production at different time points 4, 8 and 24 hours in vitro. Rate of individual gas formation (mmol/hr) was calculated from equation 6…….. 138
Table 6.2: The effect of donor diet on net H_2 mmol, CH_4 mmol and CO_2 mmol production at different time points 4, 8 and 24 hours in vitro. 139

Table 6.3: The effect of donor diet on cumulative H_2 mmol, CH_4 mmol and CO_2 mmol production at different time points 4, 8 and 24 hours in vitro. 140
Table 6.4: The effect of added sulfite in vitro on total gas and cumulative gas (mL) production at different time points 4, 8 and 24 hours in vitro. 141
Table 6.5: The effect of added sulfite in vitro on net H_2 mmol, CH_4 mmol and CO_2 mmol production at different time points 4, 8 and 24 hours in vitro. 142
Table 6.6: The effect of added sulfite in vitro on cumulative H_2 mmol, CH_4 mmol and CO_2 mmol production at different time points 4, 8 and 24 hours in vitro. 143
Table 6.7: The effect of donor diet in vitro on acetate mM, propionate mM, butyrate mM, A:P, and total VFA mM concentration at different time points 4, 8 and 24 hours in vitro. 144
Table 6.8: The effect of added sulfite in vitro on acetate mM, propionate mM, butyrate mM, A:P, and total VFA mM concentration at different time points 4, 8 and 24 hours in vitro. 146
Table 6.9: Effects of donor diet and added buffer-sulfite combinations on in vitro cumulative H_2, CH_4, and CO_2 production at 4, 8, and 24 h of incubation. 147
Table 6.10: Effects of donor diet and added in vitro sulfite combinations on in vitro VFA concentrations and acetate:propionate ratio at 4, 8, and 24 h of incubation…148

LIST OF FIGURES

Figure 1.1:Pathways of methane formation from H_2/CO_2, acetate, and methylated compounds in ruminal methanogens 12
Figure 1.2: Overview of ruminal propionate production pathways and competition for metabolic hydrogen among microbial groups..... 15
Figure 1.3: Proposed effect of seaweed supplementation on the rumen microbiome and methanogenesis. 17
Figure 1.4: Schematic representation of sulfur reduction and sulfur-containing amino acid metabolism..... 20
Figure 5.2: Effect of time on (A) pH, (B) Eh, and (C) acetate mM, and (D) propionate mM in dairy lactating cow. 118

Figure 5.3: Effect of post feeding hours on (A) butyrate, (B) A:P, and (C) total VFA concentration in dairy lactating cattle. 119

Chapter 1: INTRODUCTION

Methane is a colorless and odorless greenhouse gas produced in the rumen (87%) and small intestine (13%) that has a high capacity to trap heat in the atmosphere, causing global warming (Grossi et al., 2019; Murray et al., 1976). All of agriculture in the US contributes about 10% of the total greenhouse emissions, and livestock enteric fermentation contributed 2.87% of the total greenhouse effect over 100 years in the USA (US-EPA, 2024). In ruminants, enteric methane not only contributes to global greenhouse gas production but also causes the loss of 10-12% of the dietary metabolic energy of the feed, resulting in the reduction of feed efficiency in the livestock sector (Van Nevel and Demeyer, 1996). For this reason, reducing enteric methane while improving efficiency of energy utilization is an important goal in ruminant nutrition.

The rumen hosts a complex community of microbes such as bacteria, archaea, protozoa, and fungi which interact with each other to degrade and ferment plant fiber and nutrients to produce valuable end products (Stergiadis et al., 2021). During carbohydrate fermentation, reducing cofactors (NADH) are generated and metabolic hydrogen is released. Under anaerobic condition this process allows microbes to produce acetate and butyrate from plant fiber. To maintain the redox balance and continuation of carbohydrate fermentation, the released hydrogen must be utilized by the microbes (Kelly et al., 2022) or transferred among organisms or released as H_2 gas (Bryant et al., n.d.)

Among several pathways, because of the strong thermodynamic favorability and high affinity for H_2, methanogenesis is dominant over other hydrogen-utilizing microbes and therefore it keeps the H_2 pressure low (Van Lingen et al., 2016). Propionate from lactate and succinate, acetate via reductive acetogenesis, sulfide via sulfate reduction and

ammonia through nitrate all utilize metabolic H_2 (Haque, 2018). Propionate production is an important alternative hydrogen sink that competes with methanogenic populations for available hydrogen, where 1 mole of propionate production requires 1 mole of H_2 (1 H_2 = 2 [H]) (K. Wang et al., 2018). Likewise, acetogenic pathways may also serve as alternative sinks by utilizing 4 moles of H_2 for acetate formation via the reductive acetyl-CoA (Wood-Ljungdahl) pathway (Ragsdale & Pierce, 2008). Even though these pathways exist in the rumen, they are not favorable under normal condition. Inculcating propionic bacterium such as *Propionibacterium acidipropionici* P169 did not change gas and VFA parameters (Alazzeh et al., 2014). Similarly, Le Van et al. (1998) reported that H_2 concentration was about 400 ppm under normal conditions, whereas it increased to 4800 ppm when methane inhibitors were added, which allowed acetogenic bacteria to be detected. This suggest that H_2 can be used more efficiently in alternative pathways when methane production is inhibited. Therefore, enhancing competing hydrogen-utilizing pathways would offer an important opportunity to suppress methane formation and improve the energy efficiency of ruminant production.

Several methane mitigation strategies have been evaluated to alter the ruminal fermentation including feed management, dietary supplements, selective breeding, immunological approach, and feed additives. Among these, red algae, probiotics, sulfite and acetate have been studied to investigate their role in modulating the rumen fermentation in desired directions. Red algae and sulfite are mainly known as methane inhibitors because they interfere with methane producing enzymes (methyl co-enzyme M reductase) and often increase H_2 accumulation in the rumen (Li et al., 2025; Van'nevel et al., 1970) . This increase in H_2 may shift fermentation toward other pathways, especially

those related to propionate formation (Ungerfeld, 2015c). Probiotics, especially lactic acid bacteria (LAB), may also help modify rumen fermentation (Astuti et al., 2022). These bacteria do not directly use much hydrogen, but they can produce metabolites such as lactate, which may support propionate production through the activity of other rumen microbes. Acetate, although it is one of the main end products of rumen fermentation, has also been reported to affect methane production and increase propionate concentration in vitro (Scott, 2022). In addition, acetate is an important precursor for milk fat synthesis and may also help improve milk production (Lin et al., 2022).

Despite growing interest in feed additives like these, most have been evaluated individually, and limited information is available on the fate of excess hydrogen and its use in alternative pathways under conditions of methane inhibition. It is also still unclear whether changes in ruminal fermentation induced by these feed additives in vitro can be replicated in animal trials with the expected outcomes. Because in vitro studies are conducted under controlled conditions, host additive interactions cannot be observed. Therefore, evaluating sole additives or combination of two or more additives is required and it is necessary to replicate in vitro results in vivo (especially gas, VFA, and milk) is necessary to better understand both the mechanistic effects and the limitations of methane-mitigating feed additives.

The objectives of the present study were to assess the efficacy of feed additives such as red algae, probiotics, sulfite, and acetate on methane production and volatile fatty acid production in vitro and to assess the efficacy of the same feed additives in vivo on animal performance characteristics such as dry matter intake, body weight gain, milk yield, and milk composition, and also on ruminal fermentation characteristics such as

VFA, gases, pH, and Eh. Therefore, this study aimed to compare the in vitro and in vivo responses in ruminal parameters and to identify the limitations associated with in vivo studies.

Literature review

Carbohydrate metabolism and rumen environment

Ruminants contain a unique foregut that plays a role in the degradation and fermentation of ingested plant biomass. Complex microbial communities act on simple carbohydrates (sucrose, fructans, and starch) and complex structural carbohydrates (pectin, cellulose, and hemicellulose) to break them down into monomers (e.g., glucose) (Houtert, 1993). Fiber fermentation relies on fibrolytic enzymes in an anaerobic environment. Ruminants do not produce fibrolytic enzymes but instead depend on microbial consortia. Each cell wall component requires specific enzymes to be broken down into simpler forms. Cellulose degradation requires endo-β-1,4-glucanase, cellobiohydrolase, and β-glucosidase; similarly, hemicellulose breakdown requires endoxylanase, β-xylosidase, and glucuronoxylan hydrolases (Terry et al., 2019).

In the rumen, different bacteria, protozoa, and fungi ferment these simpler forms of carbohydrates (hexoses) into short-chain fatty acids, which are precursors of energy in ruminants, and release CO_2, H_2, methanol, methylamines, and methyl sulfides as by-products (Ungerfeld and Kohn, 2006). Glycolysis is the most common pathway used by rumen microbes for hexose metabolism, producing a three-carbon substrate known as pyruvate, along with ATP and NADH. NADH acts as an electron donor in redox reactions and is converted to NAD+, which is essential for glycolysis to continue and for microbial metabolism to be maintained (Van Lingen et al., 2016). Pyruvate is a precursor

of several volatile fatty acids. Acetate, butyrate, and propionate are the major volatile fatty acids of hexose fermentation, and each of these VFAs is produced through a specific biochemical pathway (Houtert, 1993). Moreover, carbohydrate fermentation in the rumen by microbes not only produces pyruvate and VFAs, but there are several other microbial groups that produce succinate, lactate, hydrogen, and formate (Van Houtert, 1993).

Thermodynamics and kinetics in ruminal fermentation:

The rumen is a complex ecosystem in which microbes utilize different biochemical pathways to produce various fermentation products. Studies have shown that rumen fermentation and chemical reactions are controlled by both thermodynamics and kinetics (Kohn et al., 2000). Thermodynamics describes the direction and stability of reaction pathways, whereas kinetics explains how quickly a reaction occurs in the presence of reactants. According to the second law of thermodynamics, a reaction proceeds in the forward direction until the concentration of products becomes sufficiently high relative to that of reactants, at which point the reaction may proceed in the reverse direction (Jin & Bethke, 2007). Therefore, several studies have proposed thermodynamic modeling to predict fermentation products by evaluating the biochemical pathways.

Ruminal fermentation releases energy to its surroundings, either utilized for ATP generation or lost via heat dissipation to the environment. Ungerfeld and Kohn (2006) explained that the system's Gibbs energy loss determines the direction of the biochemical reaction, as the negative change in Gibbs energy in the system drives the reaction in the forward direction. Because the reaction from A to B is considered thermodynamically feasible or spontaneous when Gibbs energy is negative ($\Delta G<0$), positive and zero values of Gibbs energy signify unfavorable and equilibrium states, respectively. In the rumen,

forming VFAs, propionate, acetate, and butyrate from pyruvate is spontaneous and has negative ΔG (Ungerfeld and Kohn, 2006). The Gibbs energy change (ΔG) during the conversion of pyruvate to acetate, butyrate, and propionate are -318 k, -318 kJ, and -322 KJ per mole of substrate, respectively when not accounting for ATP production. Since all the end products of hexose fermentation have a negative ΔG, the reaction takes place in the forward direction. Likewise, the ΔG for methanogenesis in typical ruminal conditions is -67.9 kJ per mole of methane, which favors the utilization of metabolic hydrogen to produce methane by methanogens (Beauchemin et al., 2020). Besides thermodynamics, kinetic enzymes control the metabolism pathways in the rumen. In a thermodynamically feasible reaction, enzyme kinetic plays a significant role in fermentation in which substrate concentration controls the rate of product formation.

Enzyme kinetics is a part of chemical kinetics that involves biological catalysts; it depends on the concentration of the catalyst and ambient temperature in which the chemical reaction occurs inside the rumen. In enzyme kinetics, a protein molecule (enzyme) plays a significant role in the reaction acceleration due to the presence of active sites that react with the substrate. Mathematically, the rate of initial reaction in enzyme kinetics can be formulated based on the relationships between enzyme (E), substrate (S), product (P), and time (T). Michaelis-Menten proposed $V_0 = V_{max}$ x [S]/ K_m + [S]. Vmax represents the maximum reaction velocity, and K_m is the forward catalytic constant. Several researchers have found that dose levels of enzymes significantly affect reaction rate. Miller et al. (2008) reported that the externally provided enzymes increased apparent forage quality by increasing the reaction rate between substrate and enzymes.

Fatty acid production and utilization

Ruminal fatty acids are the end products of anaerobic fermentation of organic matter in the rumen. These short-chain carbon molecules are a precursor for several other pathways that balance the rumen ecosystem and animal productivity. Several species of microorganisms metabolize the intermediary product of fermentation, eventually producing VFAs in different concentrations via specific metabolic pathways. Acetate accounted for a major portion of the total VFA, followed by propionate and butyrate in the rumen. In ruminal conditions, VFA production accelerated after 15 minutes of animal feeding (Whitelaw et al., 1970). Moreover, feed intake and microbial composition affect the relative proportion and concentration of the major VFA in the rumen (Murphy et al., 1982; Sutton, 1985). Zhang et al. (2017) found that the composition of the fibrous and starch diet (non-structural carbohydrate) improved in vitro fermentation rate and increased the production of total volatile fatty acids. Oba (2011) reported that supplementing sugars to dairy cow diets may enhance butyrate production. Animals utilize these VFAs once absorbed or transported across the ruminal epithelium via ruminal and portal veins to the destination, the liver, extrahepatic cells, and mammary epithelial cells.

The dominant VFAs like acetic, propionic, and butyric acids are utilized to produce ATP during the intermediary metabolism and these three short-chain fatty acids contributed 70 % of the total metabolic energy to ruminant (Bergman, 1990; Dijkstra, 1994). Acetate is oxidized in various cells throughout the animal's body to produce ATP (Hanson & Ballard, 1967), and it also serves as a major precursor for lipid synthesis in ruminants. In lactating cattle, a substantial amount of acetate is taken up by mammary

epithelial cells, where it is converted to acetyl-CoA and used for de novo fatty acid synthesis (Urrutia & Harvatine, 2017). This process produces short- and medium-chain fatty acids, which contribute to milk fat synthesis. Propionic acid is metabolized, especially in the liver, to produce glucose via gluconeogenesis and it support in the milk production in lactating dairy cattle (Van Houtert, 1993). Similarly, butyric acid is mostly used by gut tissues as an energy source(Baldwin et al., 1992) .

Production and fate of metabolic hydrogen in the rumen

In the rumen, approximately 80% of hexose is converted into short-chain fatty acids, and the production of acetic acid and butyric acid leaves excess hydrogen in the rumen (Czerkawski, 1970). In other words, the incomplete oxidation of one hexose molecule to acetate and butyrate produces eight and four molecules of metabolic hydrogen, respectively. If hydrogen is not utilized, or is underutilized, by other microbes, feed fermentation in the rumen may be impaired (Czerkawski, 1970). Kelly et al. (2022) It has been reported that the accumulation of H_2 in the gut affects the regeneration of electron carriers (NAD^+/NADH) during fermentation, which may influence microbial growth and metabolism. In contrast, a low level of metabolic hydrogen in the rumen provides favorable conditions for the complete fermentation of hexoses, leading to the production of adenosine triphosphate (ATP) and an optimal growth environment for microbes (Schulman, M. D. & Valentino, 1976). Moreover, the prevailing pathway of glucose fermentation determines the concentration of metabolic hydrogen in the rumen. Hydrogen is present in the ruminal environment in either dissolved or gaseous form. Microbes readily produce or remove it through several metabolic pathways, resulting in various end products.

Interspecies transfer of hydrogen is one of the major routes for hydrogen removal in the rumen. The total hydrogen level is relatively low compared with other enteric gases and is difficult to detect in the headspace. It has been reported that headspace and dissolved hydrogen concentrations are 737 μM and 0.1-50 μM, respectively (Janssen, 2010a). Hungate (1967) reported that when cows were fed alfalfa hay, the concentration of dissolved hydrogen ranged from 0.6 to 1.3 μM. A low concentration of hydrogen in the rumen indicates the utilization of metabolic hydrogen by a hydrogen sink, primarily methanogens. Dissolved hydrogen is mainly available for the reduction of carbon dioxide to methane and is in equilibrium with ruminal headspace hydrogen (M. Wang et al., 2016). The concentration of metabolic hydrogen in the rumen determines the fermentation and methane production (Janssen, 2010a). Methane production is one of the major pathways for hydrogen removal in ruminants. Moreover, limited information is available on alternative hydrogen sinks in the rumen. Further investigation is needed to identify alternative sinks that can be utilized or modulated to improve animal production.

Methanogens and Methanogenesis:

Methane is a by-product of enteric fermentation in the rumen, which is expelled through burping. It is a potent greenhouse gas causing the global temperature to rise, threatening the sustainability of natural ecosystems. Over the 20 years, methane has 80 times the global warming potential than equivalent mass of carbon dioxide (Black et al., 2021). Although methane is readily destroyed in the atmosphere, its effect is typically calculated compared to other greenhouse gases by determining the impact over 100 years. Methanogenic archaea perform methanogenesis, resulting in methane formation in the rumen's anaerobic condition. Hydrogenotrophic, acetotrophic, and methylotrophic

methanogens remove the fermentation by-products such as hydrogen (H_2), carbon dioxide (CO_2), formate, methanol, and methylamines by reducing them into methane (Figure 1. 1). The hydrogenotrophic methanogens produce the major portion of the methane in the rumen after reducing CO_2, H_2, and acetate. In general, the variability of methanogens is much lower than that of bacterial species, but methanogenesis is the thermodynamically most favorable pathway for methane production. The rumen contains approximately 10^7 to 10^9 archaeal cells/mL, which is lower than the bacterial population of 10^{10} to 10^{11} cells/mL (Pereira et al., 2022). Janssen and Kirs (2008) reported eight different species of methanogens: *Methanobacterium formicicum, Methanobacterium bryantii, Methanobrevibacter ruminantium, Methanobrevibacter millerae, Methanobrevibacter olleyae, Methanomicrobium mobile, Methanoculleus olentangyi, and Methanosarcina barkeri* are the methanogens exist in the gut system of ruminants. Amongst them, *Methanobrevibacter sp.* is the primary hydrogen sink, which scavenges one molecule of CO_2 and four molecules of hydrogen to reduce CO_2 to methane ($CO_2 + 4H_2 >>>> CH_4 + 2H_2O$) (Island et al., 2007).

Formate is produced in a very small amount in the rumen, which is immediately utilized by methanogens to produce methane (Hungate et al., 1970). Formate is produced as an intermediate during acetate formation and has a high potential to be converted into H_2 and CO_2. The formate pathway contributes approximately 18% of the total methane produced in the rumen (Y. Liu & Whitman, 2008; (Hungate et al., 1970). Another group of methanogens, such as *Methanosarcina* and *Methanosaeta*, uses the acetoclastic pathway to convert acetate into methane (Y. Liu & Whitman, 2008). Initially, acetate-oxidizing bacteria break down acetate into a carboxyl compound and oxidize it to CO_2,

which then enters the hydrogenotrophic pathway to form methane (Demirel & Scherer, 2008). The same bacteria have the ability to reverse the reaction and produce acetate using CO_2 and H_2 as substrates. Moreover, this bidirectional reaction occurs under two different conditions: when the concentration of H_2 is high (≥500 Pa), the acetogenesis pathway is favored, whereas at a low H_2 concentration (≤40 Pa), acetate oxidation occurs (Demirel & Scherer, 2008). Similarly, the methyl group of methanol and methylamine provide substrates for *Eubacterium limosum* and *Methanosarcina* spp. to convert into methane (Tapio et al., 2017). *Methanosarcina* spp. is the primarily dominant utilizer of methanol that utilizes methanol to grow itself and release methane as a by-product (Genthner et al., 1981).

Besides contributing to global climate change, methanogens also have a negative impact on fermentation, metabolism, and animal performance. Ruminal methane release is associated with a loss of metabolic energy, which reduces dietary efficiency and ultimately increases production costs. Evidence show that the intracellular and intercellular movement of metabolic hydrogen plays a significant role in determining the fate of ingested feed or its monomers. Redirecting metabolic hydrogen toward beneficial sinks, thereby inhibiting methane-forming pathways, could improve the utilization of ruminal fermentation products, enhance animal performance, and reduce environmental risk.

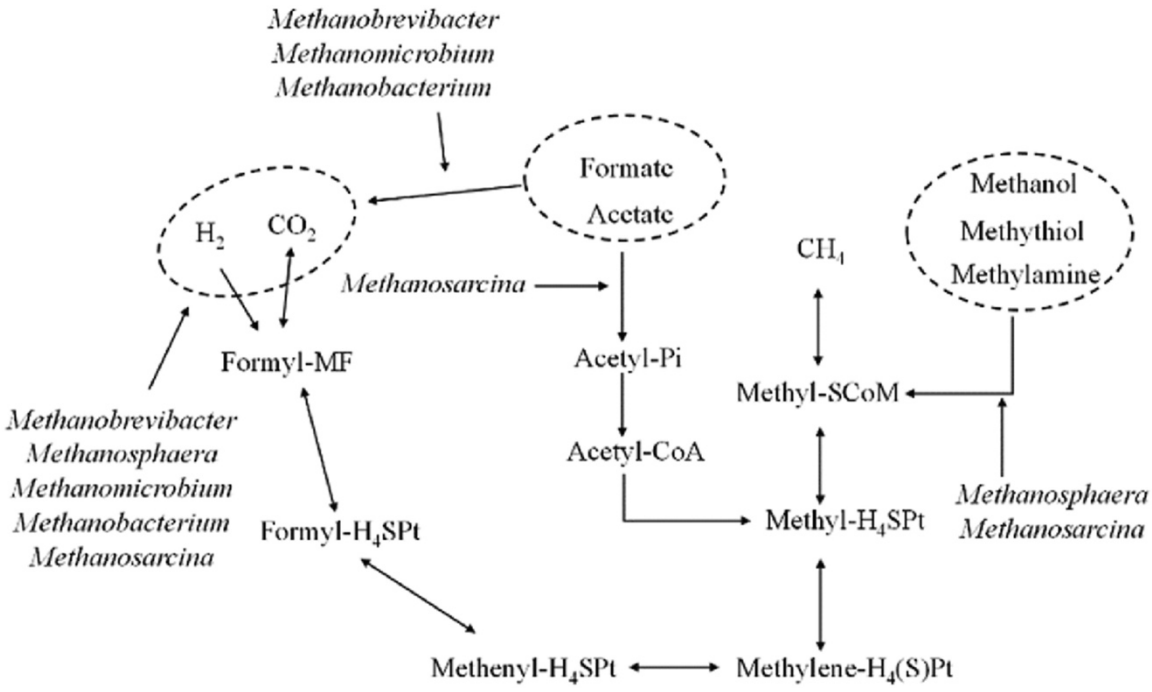


Figure 1. 1:Pathways of methane formation from H_2/CO_2, acetate, and methylated compounds in ruminal methanogens *(Zhao & Zhao, 2022)*.

Alternative hydrogen sink

Reductive acetogenesis pathway

Reductive acetogenesis is an alternative biochemical pathway, also known as the Wood-Ljungdahl pathway, that utilizes 2 moles of carbon dioxide and 4 moles of hydrogen to produce acetate as an end product. Acetogenic bacteria primarily metabolize carbon dioxide and hydrogen, but they can also oxidize pentoses, hexoses, alcohols, formic acid, and methyl groups (Yang, 2018). *Lachnospiraceae*, *Clostridiaceae*, and *Ruminococcaceae* are the major family of acetogens found in the rumen (Gagen et al., 2015). Even though methanogens and acetogens commonly use CO_2 and H_2 as major substrates for their energy source, the lower threshold of methanogens outcompete the headspace hydrogen and makes it less available for acetogens to form acetate. Ungerfeld

and Kohn (2006) reported that acetate formation from H_2 and CO_2 is not thermodynamically feasible in the normal ruminal environment unless methanogens are inhibited, or hydrogen level is increased. Inhibiting the methanogenesis leads to more headspace hydrogen that could make the acetogenic pathway thermodynamically feasible. One way to control methanogens is by adding inhibitors; the second would be by lowering the pH. Because methanogens are pH sensitive, the population decreases when the pH drops less than 6.5, which makes acetogens more thermodynamically feasible to utilize H_2 and CO_2 (Ungerfeld, 2013). However, in normal conditions, adding acetogenic bacteria could not affect the methanogenic activity unless some inhibitors inhibit methanogens Lopez et al. (1999). Contrary to previous studies, recent studies have found that some acetogens from cows and sheep act as hydrogenotrophic and can compete with methanogens (Joblin, 1999). Adding these bacteria in a ruminal environment alleviates methane production and provides a natural way to dispose of hydrogens for better use in animal production.

Propionate pathway

As most microbes utilize H_2 and CO_2 as energy sources to produce their end products, inhibiting or limiting unproductive pathways could enhance animal performance. Propionate is the major glucose precursor in ruminants; therefore, redirecting metabolic H_2 toward propionate production is considered an efficient strategy to improve feed efficiency and reduce enteric methane emissions. Propionate is primarily formed during ruminal fermentation through the fumarate-succinate and acrylate pathways (Figure 1. 2); both pathways utilize four metabolic hydrogens to reduce oxaloacetate and pyruvate, respectively, to propionate as the final product (Choudhury et

al., 2022). These pathways serve as alternative means of disposing of hydrogen in the rumen headspace. Carbohydrates and lactate are the major substrates used by bacteria such as *Selenomonas ruminantium* and *Megasphaera elsdenii* to produce propionate via the acrylate pathway, whereas cellulolytic bacteria such as *Ruminococcus flavefaciens* and *Bacteroides succinogenes* produce succinate, which is rapidly converted to propionate through the involvement of two rate-limiting enzymes: succinate-CoA synthetase and propionate-CoA transferase (Scheifinger & Wolin, 1973; Wang et al., 2020).

Sugar- and starch-utilizing bacteria have been reported to produce more propionate; thus, propionate production can be favored by manipulating one or both of the acrylate and succinate pathways. Several studies have demonstrated that high-concentrate diets enhance the propionate pathway and produce more propionate than other VFAs (Scott & Kohn, 2022; Yi et al., 2022). Wang et al. (2024) reported dairy cows in high grain (60:40) diet significantly increased the molar proportion of propionate as compared to the low grain diet. It had been reported that high concentrate favored propionate pathways due to the growth of firmicutes over Bacteroidetes and of *Methanobrevibacter* over *Methanomassiliicoccus* that results in shifting of H_2 toward Propionate (Wang et al., 2018). Besides feed manipulation, several methane-mitigating feed additives have shown prominent results in reducing methane and redirecting the available hydrogen toward VFA production. These feed additives include marine seaweeds, sulfite, and acetate.

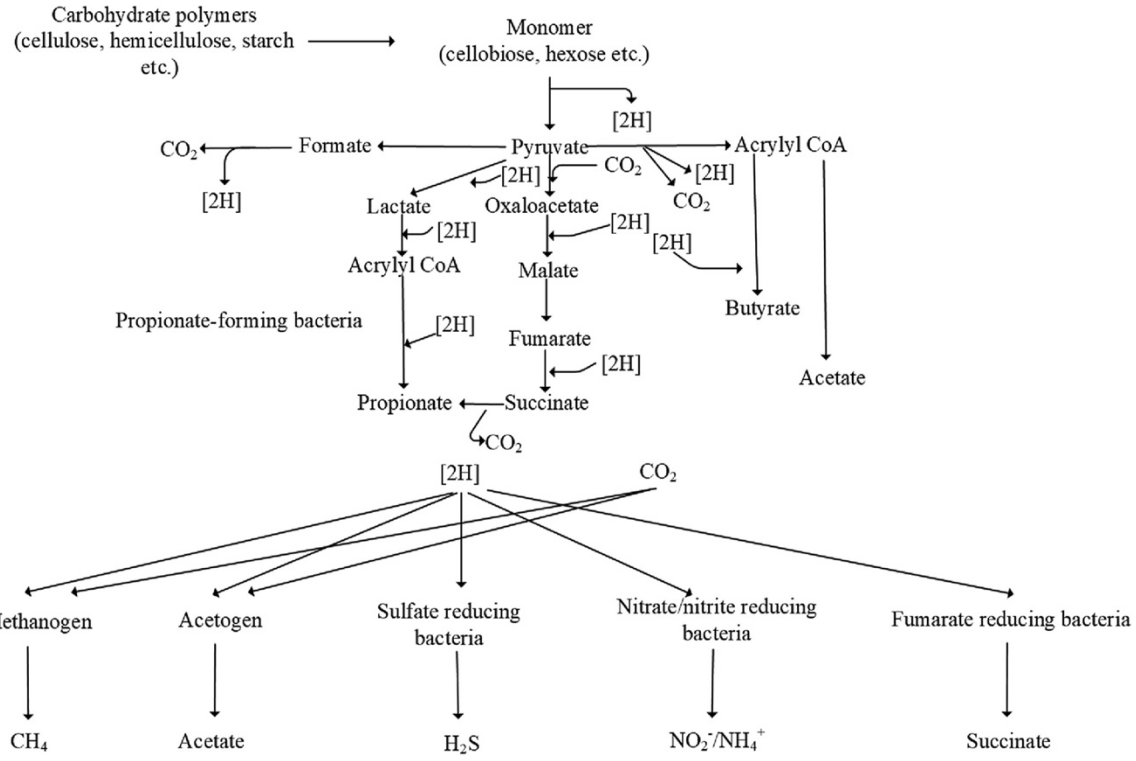


Figure 1. 2: Overview of ruminal propionate production pathways and competition for metabolic hydrogen among microbial groups *(Zhao & Zhao, 2022).*

Feed additives

Seaweeds

Human and animal diets have included seaweeds since ancient times. There are two major types of seaweed: macroalgae and microalgae. Brown, red, and green algae are the main forms of macroalgae. Nutritionally, seaweeds are good sources of protein, minerals, and several bioactive secondary metabolites. However, the final nutrient content of algae depends on the species, time of collection, and growth environment. For example, brown, red, and green seaweeds contain protein at 5-12%, 18-30%, and 12-15% of DM, respectively (Makkar et al., 2016). Therefore, brown algae are nutritionally less important than red and green algae, as brown algae contain less protein and many

minerals (Makkar et al., 2016). Despite all, the availability of bioactive compounds (phenolic and halogenated compounds) makes this feed a special additive as it reportedly helps reduce methane and improves VFA synthesis.

Bromoform, dibromochloromethane, bromochloroacetate, dibromoacetate, chloromethane, and bromomethane are halogenated compounds that act as methane analogs. These bioactive compounds are released into the culture medium and exhibit anti-methanogenic properties similar to those of bromochloromethane (Denman et al., 2007). These methane analogs from algae inhibit the carbamide-dependent methyl-transfer reaction, primarily giving methane as an end product from several methanogenesis pathways (Tedeschi, 2021).

Red seaweeds such as *Asparagopsis taxiformis* and *Asparogopsis armata* contain the highest percentage of bromoform (1.73 mg/ DM) and then followed by dibromochloromethane ($CHBr_2Cl$; 0.158 μg/g DM), bromochloroacetate ($C_2H_2BrClO_2$; 0.088 μg/g DM), and dibromoacetate ($C_2H_2Br_2O_2$; 0.009 μg/g DM) (Min et al., 2021). In both in vivo and in vitro studies, these seaweeds have shown promising results in reducing methane at different inclusion levels. An in vitro study using ruminal inoculum from Brahman steers (*Bos indicus*) showed a significant reduction in total gas production and complete elimination of enteric methane emissions when the inclusion rate was greater than 2% of OM, whereas inclusion rates below 1% of OM had minimal effects on total gas and methane production (Kinley, De Nys, et al., 2016). As stated earlier, inhibition of enteric methane emissions provides an opportunity to grow alternative pathways such as propionate; therefore, Kinley et al. (2016) reported that propionate level improved at the increasing doses of the red algae and obtained higher propionate:

acetate ratio. Similarly, another study on beef steer showed that in high forage diet, methane production was reduced by 32% and 59%, where H_2 accumulation in the rumen increased by 178% and 361% in the low (0.25% of OM) and high (0.5% of OM) inclusion of *Asparogopsis taxiformi,* respectively (Roque et al., 2021). Red algae seem to be the prominent effective source to be added to the animal diet to reduce enteric methane emission (Figure 1. 3); however, its long-term viability regarding animal health productivity is yet to be tested. As seaweeds are rich in mineral content that might also result in the potential human health risk in long term, however study needs to be done to understand the residual effect of seaweeds on human and animal health.

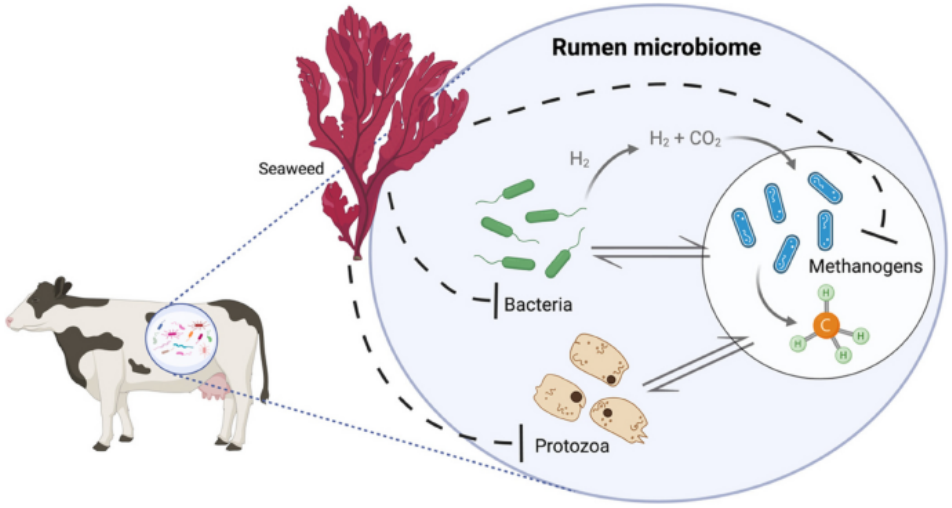


Figure 1. 3: Proposed effect of seaweed supplementation on the rumen microbiome and methanogenesis (McGurrin et al., 2023).

Probiotics

Animals, especially ruminants, contain billions of microorganisms, and it is believed that microbial population and diversity can be modified for better health and production (O'hara et al., 2020). Using probiotics as feed supplements in animal diets is one of the most utilized methods to improve the gut microflora and overall performance

of animals. Those non-pathogenic and nontoxic microorganisms consist of living cells, can survive in the host gut, can be isolated from the same species as the host, and eventually benefit the host are considered probiotics (Collins & Gibson, 1999). In a real sense, probiotics are the living microorganisms being utilized to improve the host's health (Hill et al., 2014). In the ruminant industry, probiotics are mainly utilized to improve fermentation and reduce the energy loss of feeds while improving their end products (meat and milk).

Studies have found that lactic acid (LAB), propionic acid bacteria, and acetic acid bacteria are major probiotics used in the animal industry (Sun et al., 2021). Similarly, acetic acid bacteria are acetogens, which are capable of reducing the number of methanogens in the gut (Kim et al., 2018). Lactic acid bacteria are natural components of the gut microbiota that can manipulate the gut microbial population while preventing the growth of pathogenic bacteria (Signorini et al., 2012). LAB are a gram-positive bacterium, and the ferment carbohydrates to produce lactic acid as an end product in the rumen (Doyle et al., 2019). LAB can be easily isolated from the rumen; among several species, *Lactobacillus ruminis* and *Streptococcus equi*

nus are the major inhabitants of the rumen (Stewart, 1992). However*, Bacillus sp.* is mostly a lactic acid bacterium in nature, which showed the potential to be utilized as probiotics as these bacteria can produce essential enzymes in the gut that support the digestion of feed (Sorokulova, 2013). Also, LAB and their metabolites are used to mitigate the methane production in three possible ways: 1) shifting rumen fermentation towards propionate formation, 2) directly inhibiting methanogens fermentation products such as bacteriocin, 3) inhibiting microbes that produce substrate for methanogens (Doyle

et al., 2019). Jeyanathan et al. (2016) reported that out of three selected strains of LAB, only *Lactobacillus pentosus* D31 was able to decrease the methane production by 13% in sheep. Similarly, supplementation of *L. plantarum* (strain U32) had the lowest CH4 production in vitro with improved propionate production (Astuti et al., 2018). The effect of LAB on rumen fermentation has not been consistent across studies, as it appears to depend on the specific species or strain used. In addition, no studies have yet investigated the combined use of LAB with other methane inhibitors. Therefore, the potential role of LAB as an alternative electron sink under methane-inhibited conditions needs to be evaluated.

Sulfur compounds

Sulfur is essential for living cells as it is present in the amino acids, hormones, vitamins and co-enzymes. In addition, sulfur is needed for bacterial growth and metabolic activities in some ruminal cellulolytic bacteria (Drewnoski et al., 2014). Also, Sulfur and nitrogen ration is essential for nitrogen metabolism, the imbalance in the ratio can results in an inability to synthesize enough S-containing AA like cystine and methionine and microbial protein (Zhao et al., 2020). Sulfur in rumen metabolized via assimilatory and dissimilatory class of bacteria, where, initial two steps are similar.

Assimilatory bacteria incorporate sulfide into sulfur-containing amino acids (mainly cysteine and methionine) and into sulfur-containing cofactors such as biotin and pantothenic acid via assimilatory sulfate reduction, in which sulfate is reduced to sulfide for biosynthetic purposes (Bradley et al., 2011). In contrast, dissimilatory sulfate-reducing bacteria use sulfate as a terminal electron acceptor, sulfate is transported into the cytoplasm, activated to adenosine-5′-phosphosulfate (APS), and sequentially

reduced to sulfite by APS reductase and to sulfide by sulfite reductase, with ferredoxin and NAD(P)H serving as major electron donors; the resulting sulfide is released predominantly as hydrogen sulfide (H_2S) (Saito, 2004) (Figure 1. 4). Although SRB population is small in number but plays a important role in sulfur digestion of diets containing sulfur.

It was reported that, infusion of the sulfite as precursor of sulfide directly affected the methanogen population in vitro and in vivo and increased propionate production in wethers (Van Nevel et al., 1970a). Also, sulfite inclusion at the rate of 160 g in the daily ration of lactating Holstein cows increased the molar percentage of propionate by 5.7% where acetate production decreased by 10% (Krabill et al., 1969a). In the same study, total milk production, milk fat and dry matter intake was not affected by the addition of the sulfite. However, addition of sulfur compound more than requirement can cause serious health issues such as polioencephalomalacia (PEM), a neurological disorder (Dore & Smith, 2017). Thus far, a single study on the cattle has provided limited information on the fermentation parameters of the rumen, therefore this project will try to explore the effect of the sulfite on the dairy cattle with different dietary regimes.

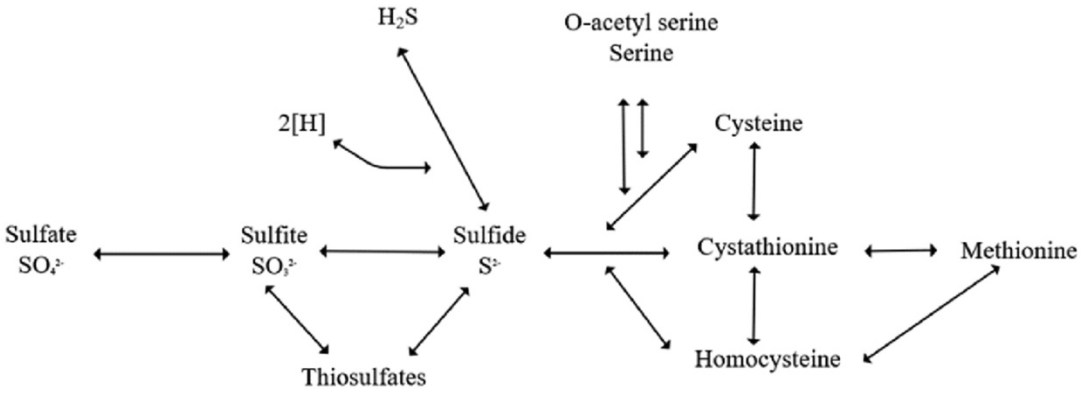


Figure 1. 4: Schematic representation of sulfur reduction and sulfur-containing amino acid metabolism *(Zhao & Zhao, 2022)*

Sodium Acetate

Fatty acid synthesis in the mammary tissue of ruminants differs significantly from that in non-ruminant tissues. Because the ruminant mammary tissue has a limited ability to use glucose, pyruvate, and glycerol for fatty acid synthesis, as these metabolites produce mitochondrial acetyl-CoA (Laliotis et al., 2010). The low levels of ATP-citrate lyase and NADP-malate dehydrogenase activities in mammary tissue cannot effectively utilize mitochondrial acetyl-CoA, which hinders the ability to use metabolites that create mitochondrial acetyl-CoA for fatty acid synthesis. Therefore, ruminants evolved to utilize the acetate and β-hydroxybutyrate (BHBA) as a carbon source for fatty acid synthesis in the mammary epithelial tissues (Na & Guan, 2022).

In the process of milk fat synthesis, acetate and β-hydroxybutyrate are absorbed into blood vessels and transported through circulation to the mammary epithelial cells. In these cells, they are activated by acyl-CoA synthetase (ACSS), which converts them into acetyl-CoA and β-hydroxybutyryl-CoA (BHBA), respectively. In the cytosol, de novo fatty acid synthesis takes place through the actions of acetyl-CoA carboxylase (ACACA) and fatty acid synthase (FASN), resulting in the production of short- and medium-chain fatty acids. Previous reports suggested that infusion of acetate increased the fat concentration by 0.25 %/kg of acetate infusion and yield increased by 75.5 grams/day/kg of acetate (Maxin et al., 2011). Additionally, the addition of the sodium acetate in the ruminal in vitro study reported that it increases the proportion of propionate while methane production dropped (Scott, 2022). However, limited research has evaluated the effects of acetate supplementation on animal performance, including milk yield, milk

composition, and body weight gain, to better understand energy partitioning within the animal. Therefore, further investigation is needed to validate the in vitro findings of sodium acetate supplementation under in vivo conditions and to determine its effects on animal performance and ruminal fermentation parameters.

Chapter 2: Evaluation of the combined effect of red algae (*Asparogopsis taxiformis)* and probiotics (*Lactobacillus planturum*) on fermentation parameters in vitro

ABSTRACT

In ruminants, methanogenesis serves as a sink for the disposal of hydrogen gas, contributing to methane production. Inoculating lactic acid bacteria (LAB) in combination with red algae could provide an alternative hydrogen sink, promoting the production of volatile fatty acids (VFAs) from hydrogen and CO_2. However, the combined effects of gram-positive bacteria and the anti-methanogenic properties of red algae have not been well studied. Therefore, the objective of this study was to evaluate the impact of adding lactic acid bacteria (*Lactobacillus plantarum*) and red algae on ruminal fermentation parameters, with particular emphasis on gas production, using an in vitro system. Rumen fluid was collected from a fistulated, non-lactating Holstein cow prior to the early morning feeding. The rumen fluid was processed in the laboratory under a continuous CO_2 flush and mixed with reduced in vitro media at a 50:50 ratio by volume. Timothy hay (0.76 g) was added to Wheaton bottles (150 mL; n = 20), followed by the addition of red algae (2% of dry matter) to the designated bottles. Subsequently, 30 mL of the rumen fluid mixture was added to each bottle, and 0.5 mL of media with or without Lactobacillus plantarum (LAB; 10^7-10^8 CFU/mL) were anaerobically added to the respective bottles. Bottles were randomly assigned to one of the following treatments: 1) Control, 2) Algae, 3) LAB + Algae, and 4) LAB. Gas samples were collected from each bottle at 0, 4, 24, 48, and 72 hours of incubation. Data were analyzed using a completely randomized design with repeated measures over time in JMP® Pro 17.2.0 (SAS Institute Inc.). Methane fraction and cumulative methane production were lower in the algae and mixed treatments than in the control and LAB-only treatments from 0 to 72 hours ($P < 0.05$). Additionally, net methane production and the rate of methane formation

were undetectable in the algae and mixed treatments from 0 to 48 hours, whereas the control and LAB-only treatments showed higher methane production during this period ($P < 0.05$). The H_2 fraction, net H_2 production, cumulative H_2 production, and rate of H_2 formation were significantly higher in the algae and mixed treatments compared with the control and LAB-only treatments ($P < 0.05$), with differences observed primarily between 0 and 48 hours. Similarly, total gas production and cumulative gas production were lower in the algae and mixed treatments than in the control and LAB-only treatments up to 24 and 48 hours, respectively ($P < 0.05$). However, gas production in the algae and mixed treatments increased at 48 and 72 hours and exceeded that of the control and LAB-only treatments ($P < 0.05$). No differences were observed among treatments for any carbon dioxide-related parameters. The persistently lower methane production in algae-containing treatments highlights their mitigation potential; however, further validation is required to determine whether excess H_2 is redirected toward beneficial fermentation pathways, such as propionate production, in the presence of lactic acid bacteria.
Key words: bromoform, gas, methanogenesis, Seaweeds

INTRODUCTION

Seaweeds such as *Asparagopsis taxiformis* contain halogenated methane analogs (e.g., bromoform), which structurally resemble methyl-coenzyme M. The bromoform in these seaweeds primarily targets methyl-coenzyme M reductase, the enzyme that catalyzes the final step in methanogenesis (Roque et al., 2019). A previous study demonstrated that including seaweed decreased methane production by up to 99% when added at less than 2% of organic matter (OM) basis (Machado et al., 2016), whereas Angus-Hereford cross beef steers showed an 80% reduction in methane when supplemented at 0.5% OM basis (Roque et al., 2021). The inclusion of seaweed not only decreased the activity of methanogens, it also attributed increased headspace H_2 concentration. *Asparagopsis taxiformis* at 2% of OM increased hydrogen accumulation by 4.7 mL/g OM at 48 hours, and elevated H_2 levels persisted up to 72 hours in the in vitro study (Machado et al., 2018).

Adding methane inhibitors like red seaweeds increases the H_2 partial pressure, but this condition leads to important changes in the biochemical pathways as well as microbial changes (Ungerfeld, 2020). Because hydrogen is a key electron carrier in the rumen, its production and disposal play a vital role in maintaining redox balance under anaerobic conditions (Mackie et al., 2024). When hydrogen accumulates, hydrogen forming pathways in such conditions become thermodynamically infeasible; as a result, acetate production is affected (Ungerfeld and Kohn, 2006; Van Lingen et al., 2016). Additionally, microbial shifts occur where excess hydrogen is utilized by propionate producers. Previous studies have demonstrated that adding methane inhibitors increased the change in negative Gibbs energy for the incorporation of H_2 during the conversion of

acetate to propionate, resulting in more propionate formation in both continuous and batch cultures (Ungerfeld, 2015). However, the change in redox balance directly affects fibrolytic bacteria, and fiber digestibility is ultimately reduced (Firkins & Mitchell, 2023). Therefore, finding alternative hydrogen sinks, such as probiotics that utilize hydrogen, may help mitigate these issues and support more stable rumen fermentation.

In the rumen, propionate is produced mainly via the succinate and acrylate pathways. The acrylate pathway specifically requires lactate as an intermediate to generate propionate, whereas the succinate pathway directly utilizes H_2 during the conversion of fumarate to succinate and ultimately to propionate (B. Zhang et al., 2022). Schulmand & Valentino (1976) reported that under elevated H_2 levels, lactate fermentation resulted in a 4-8-fold increase in propionate production, along with a significant increase in total volatile fatty acid production. However, in the same experiment, when succinate was used as the substrate, propionate remained the main end product, but total volatile fatty acids decreased at the expense of acetate. Therefore, supplementing lactic acid bacteria (LAB) as probiotics, along with algae, may enhance propionate and total VFA production formation through the lactate-utilizing acrylate pathway.

The aim of the current study is to determine the effect of the combination of red algae (*Asparagopsis taxiformis*) and *Lactobacillus plantarum* on the production of gas. According to the study hypothesis, supplementing with red algae causes inhibition of the process of methanogenesis by affecting the functioning of methyl co-enzyme M reductase activity, thereby causing a buildup of hydrogen. When present together with lactic acid-producing bacteria, this excess H_2 will find its way into thermodynamically

favorable processes of fermentation, particularly propionate pathways. Inhibition of methane alone can lead to a buildup of H_2, potentially limiting the processes of fermentation because of the imbalance created by changes in the rumen redox balance. Hence, directing the H_2 buildup to acrylate pathways through the addition of LAB will enhance the production of propionate with deceased methane.

MATERIALS AND METHODS

Rumen fluid collection and in vitro preparation

The experimental procedures were approved by the University of Maryland Institutional Animal Care and Use Committee (IACUC#- R-MAY-25-18) ahead of the starting date of experiment. On the day of the experiment, fresh rumen fluid was collected from a non-lactating dry Holstein-Friesian cow at the university facility, approximately two hours before the morning feeding. The rumen contents were immediately transported to the laboratory and homogenized in a blender for 20 seconds. The homogenized fluid was then strained through four layers of cheesecloth and glass wool to remove particulate matter. All procedures were performed under strictly anaerobic conditions at every step.

Prior to rumen fluid collection, 0.76 g of orchard hay and red algae (2% of DM) were added to the appropriate 150-mL Wheaton bottles. The bottles were sealed with rubber stoppers and aluminum caps. Each bottle was then flushed with CO_2 using a 20-gauge inlet needle and a 22-gauge outlet needle at least for 20 minutes. A subset of bottles was randomly selected after flushing, and gas samples were collected and

analyzed using gas chromatography to verify air contamination levels and ensure that the system remained strictly anaerobic.

The in vitro media were also prepared on the same day of the experiment. After processing the rumen fluid, the in vitro media were added in equal proportion to the rumen liquid and mixed using a stir bar throughout the filling process. A total of 30 mL of the rumen fluid media mixture (50:50) was transferred into each Wheaton bottle. Then, 0.5 mL of media with or without *Lactobacillus Plantarum* (LAB; 10^7-10^8 cfu/mL) was added anaerobically to the respective bottles.

The lactic acid bacteria were obtained from preserved stock maintained in the laboratory. Then LAB was cultured in MRS broth (Sigma-Aldrich) using the in vitro media. The LAB cultures were incubated for 24 to 48 hours at 39 °C. The experimental treatments included: control (hay only), LAB only, LAB + algae, and algae only. The experiment was repeated twice, with 5 replicates per treatment in each batch culture. These bottles were placed on shaker inside an incubator at 39 °C for 72 hours.

Gas and VFA samplings

Gas and inoculum samples for gas and VFA analysis were collected at 0, 4, 24, 48, and 72 hours. Gas volume was measured using a gas-tight syringe fitted with a two-way valve and hypodermic needle. A Fortuna glass syringe fitted with a 22-gauge needle was used to collect and measure the volume of gas at each time points. Liquid samples were obtained by inverting the bottles, allowing the solid substrates to settle, and withdrawing approximately 1-1.5 mL of supernatant into 2 mL centrifuge tubes.

Gas samples were immediately analyzed for hydrogen, carbon dioxide, and methane concentrations using a gas chromatograph (GC). Liquid samples were stored in

a freezer at -20 °C. Before analysis, the samples were removed from the freezer and allowed to thaw at room temperature for 15-20 minutes. The thawed samples were then processed and analyzed for acetate, propionate, and butyrate using GC.

Gas equation

Total gas production, cumulative gas production, net individual gas production, change in individual gas production, cumulative, rate of individual gas formation were calculated based on the following equation:

$$V_t = V_{s,t} + V_h \ldots\ldots\{1\}$$

$$V_{cum,t} = V_h + \sum_{t=0}^{t} V_{s,t} \ldots\ldots\ldots\{2\}$$

$$X_t = \frac{(V_{s,t} \times C_t) + V_h (C_t - C_{t-1})}{22.4} \ldots\ldots\ldots\{3\}$$

$$X_{cum,t} = X_t + \sum_{t=0}^{t} (X_{t-1}) \ldots\ldots\ldots\{4\}$$

$$\frac{}{22.4}$$

Where,

V_t = volume of gas (mL) at time t

$V_{cum,t}$ = Cumulative gas production at time t (mL)

X_t = Net production of individual gas (CH_4, H_2 and CO_2) at time point (t) (mmol)

$X_{cum,t}$ = Cumulative individual gas production at time t (mmol)

$V_{s,t}$ = Volume of gas collected in syringe at time t, and

V_h = Volume of headspace

Vs_{t-1} = Volume (mL) of gas in syringes in previous time point

C_t = Concentration of individual gas at time t and 22.4 is the molar volume of an ideal gas at STP.

C_{t-1} = Concentration of individual gas in previous time point (t-1)

X_{t-1} = Production of individual gas (CH_4, H_2 and CO_2) at time point (t-1) (mmol)

Statistical analysis

Data retrieved from the GC system were processed and statistically analyzed using a randomized block design with repeated measures over time in JMP® Student Edition 19.0 (SAS Institute Inc.). The statistical model included four levels of treatment and five sampling time points. Data were analyzed using a one-way ANOVA, and comparisons among treatment groups were made separately at each time point. Batch was included in the model as a random effect. As the analysis was done for individual time points, the variable 'time' was used as a by variable in the JMP software, with a separate analysis done for each individual time point (0, 4, 24, 48, and 72 hr).

The statistical model was:

$$Y_{bijk} = \mu + B_b + T_i + R_{(j(bi))} + \varepsilon_{bijk}$$

where

Y_{bijk} = observed response (e.g., CH_4, H_2, and CO_2),

μ = overall mean,

B_b = random effect of batch, (that's a block)

T_i = fixed effect of treatment,

$R_{(j(bi))}$ = random effect of batch

ε_{bijk} = residual error.

Significance was declared at $P < 0.05$ and tendency towards statistical differences was declared at $0.05< P <0.10$.

RESULTS

Total gas and cumulative gas

Total gas production was lower at 4 and 24 hours in the algae and combined (LAB + algae) treatments compared with the control and LAB-only treatments. However, gas production in the algae and combined treatments increased at 48 and 72 hours and exceeded that of the control and LAB-only treatments (Table 2.1). Similarly, cumulative gas production was higher in the control and LAB-only treatments than in the algae and combined treatments at 4 and 24 hours. At 48 hours, cumulative gas production remained higher only in the control treatment compared with the other treatments.

Hydrogen

The H_2 fraction, net H_2 production, change in H_2 production, and cumulative H_2 production were significantly higher in the algae and combined treatments than in the control and LAB-only treatments up to 48 hours (Table 2.2 - 2. *6*). However, at 48 hours, the change in H_2 production was greater in the control and LAB-only treatments than in the algae and combined treatments. Cumulative H_2 production remained higher in the algae and combined treatments through 72 hours compared with the control and LAB-only treatments.

Methane

Methane concentration in the algae and combined treatments was nearly undetectable, whereas the control and LAB-only treatments exhibited higher methane concentrations up to 72 hours (Table 2.2). Similarly, net methane production were near zero in the algae and combined treatments compared with the control and LAB-only treatments up to 48 hours, except for the algae-only treatment at 4 hours (Table 2.3). Cumulative methane production (Table 2.4) was lowest in the algae and combined treatments compared with the control and LAB-only treatments and remained suppressed through 48 hours.

DISCUSSION

Gas parameters

Similarly, the effect of algae on the total gas production was observed in both algae included treatments than LAB and control. The maximum effect on reduction was observed at time point 48 hours where nearly 9% less gas was produced. This finding aligns with previous observations where total gas was reduced in *Asparogopsis taxiformis* added in vitro rumen culture in 72 hours incubation period (Kinley et al., 2016). Similarly cumulative gas production was less in the algae included treatment than control and LAB till 48 hours, where no differences were observed at 72 hours. The reduction in total gas production could be due to elevated H_2 partial pressure, as the Gibbs free energy for reoxidation of the reducing cofactor NADH becomes less favorable. Consequently, NADH cannot be efficiently re-oxidized to NAD^+, which is essential for glycolysis and carbohydrate degradation to acetate under anaerobic conditions (Tenci et al., 2024). This feedback mechanism slows the acetate-forming pathway; as a result, H_2 and CO_2

production decrease, leading to an overall reduction in total gas production. The total gas production after 48 hours started increasing in the algae and mixed treatments. This suggests that once H_2 was utilized by microbes, the inhibitory feedback effect of high H_2 partial pressure was reduced, making fermentation more feasible and resulting in greater gas production. Another possible reason could be that the diminishing methane-inhibitory effect of red algae over time, along with partial microbial adaptation to the algae, which could lower H_2 partial pressure.

Consistent with previous in semi-continuous in vitro study (Roque et al., 2019), the current study demonstrated that inclusion of *Asparagopsis taxiformis* increased H_2 production. Because methanogens are the major consumers of H_2 under ruminal conditions, suppression of methanogenic activity results in the accumulation of excess H_2 in the rumen. Accordingly, red algae drastically increased the H_2 fraction, production, and rate of formation in this study and the effect of algae lasted for 72 hours. However, under methane-inhibiting conditions, elevated H_2 partial pressure favors alternative H_2-utilizing pathways, redirecting reducing equivalents toward thermodynamically favorable reactions that lead to the production of propionate or acetate (Ungerfeld, 2015).

Red algae contain bromoform, which is expected to reduce methane emissions. In this study, 2% red algae had a significant effect on methane-forming pathways, where hydrogen- and carbon dioxide-utilizing microbes (methanogenic archaea) were drastically suppressed in the presence of algae. The fraction, production, and cumulative production of methane clearly indicate that the effect was pronounced even at a 2% inclusion level, where methane was untraceable by gas chromatography, and methane production was almost negligible up to 48 hours. This study aligns with previous findings

that red algae, especially *Asparagopsis taxiformis*, are highly effective in reducing methane emissions by up to 95% in semi-continuous in vitro rumen systems (Roque et al., 2019).

The red algae are rich in halogenated methane analogues that target the terminal steps of methanogenesis on which Methyl coenzyme M reductase (MCR) is the key terminal enzyme inhibited by the halogenated compound bromoform present in red algae. (Li et al., 2025). The anti-methanogenic compound reacts with cofactors such as vitamin B_{12}, which is crucial for transferring the methyl group to coenzyme M via methyltransferase. In addition, this compound directly binds to cofactor F_{430} of methyl coenzyme M reductase (MCR), which is responsible for releasing CH_4 at the terminal step of methanogenesis after electron acceptance (Tenci et al., 2024).

However, the effect of algae appeared to decline slightly after 48 h; therefore, methane was detected in the incubation bottles after 72 h, with no differences among treatments. This could be because, after several hours of inclusion in the rumen, some bromoform-degrading microbes begin to degrade bromoform via a dehalogenation process; therefore, the effectiveness of the algae is reduced over time (Glasson et al., 2022). The inclusion of lactic acid bacteria along with red algae had no significant effect on the methane reduction compared to sole algae.

Propionate forming pathways is second most H_2 utilizing pathway after methanogenesis therefore we hypothesized that supplementing LAB with algae would improve the H_2 acrylate pathways thereby further decreased the free H_2 availability for the methanogenesis compared to algae alone. Since we do not have results from VFA analysis, and the head space H_2 production showed no differences in between LAB and

LAB + Algae. Propionate concentrations increased in parallel with greater abundance of propionate-producing microbes such as *Prevotella* spp.; however, H_2 still accumulated, with only about 20% being incorporated into metabolic sinks and roughly 80% ultimately being released by the animal (Mitsumori et al., 2014). This finding suggests that a greater population of propionate producers does not proportionally reduce H_2 concentration. One possible explanation is that the incorporation of H_2 into propionate is thermodynamically constrained, as the reaction remains near equilibrium under methane-uninhibited conditions and only slightly negative under methane-inhibited conditions (Ungerfeld, 2015).

Summary

This study demonstrated that, the inclusion of red algae, either alone or in combination with LAB, drastically reduced enteric methane emissions even at a 2% dry matter basis with methane production was approached to nearly zero in the algae-containing treatments. Additionally, the hydrogen production increased in algae included culture with no effect on carbon dioxide production at any time point. The LAB included culture showed no measurable difference in gas production as well as methane reduction compared to control culture, showing inefficacy of the bacterial strain that being used for this study. This would suggest that LAB did not shift excess H_2 to alternative acrylate pathway as was hypothesized. Further, VFA analysis will be needed to verify if hydrogen shifted towards propionate pathways in methane inhibited condition.

Table 2.1: The effect of *Asparogopsis taxiformis*, *Lactobacillus plantarum*, and their combination on total gas at 1 atm. measured at each time step (gas in excess of bottle headspace volume was discarded after measurement) and cumulative gas (mL) production at 0, 4, 8, 24, 48, 72 hours of time interval in vitro.

			Total gas (mL)			
Treatment	**0 HR**	**4 HR**	**24 HR**	**48 HR**	**72 HR**	**SEM**
Control	120	172[a]	192[a]	125[b]	123[b]	3.3
Alga only	120	163[b]	184[b]	136[a]	128[a]	3.3
LAB only	120	171[a]	193[a]	124[a]	124[b]	3.3
Algae + LAB	120	163[b]	186[b]	135[a]	128[a]	3.3
			Cumulative gas (mL)			
Control	120	172[a]	245[a]	254[a]	255	9.4
Alga only	120	163[b]	227[b]	243[b]	251	9.4
LAB only	120	171[a]	245[a]	250[ab]	254	9.4
Algae + LAB	120	163[b]	230[b]	244[b]	253	9.4

The total gas (mL) and cumulative gas (mL) were derived using equation 1 and 2.

[a-b] Means within a column not connected by the same letter are significantly different ($P < 0.05$).

Table 2.2: The effect of *Asparogopsis taxiformis* and *Lactobacillus plantarum* on H_2, CH_4 and CO_2 concentration at different time intervals 0, 4, 24, 48 and 72 hours in vitro.

Treatment	0 HR	4 HR	24 HR	48 HR	72 HR	SEM
			H_2 Fraction			
Control	0.0001^{c}	0.0003^{b}	-0.0000^{b}	0.0003^{b}	0.0037^{b}	0.00285
Alga only	0.0003^{a}	0.0172^{a}	0.0561^{a}	0.0731^{a}	0.0689^{a}	0.00273
LAB only	0.0001^{c}	0.0003^{b}	0.0001^{b}	0.0004^{b}	0.0021^{b}	0.00298
Algae + LAB	0.0002^{b}	0.0179^{b}	0.0553^{a}	0.0708^{a}	0.0640^{a}	0.00268
			CH_4 Fraction			
Control	0.0005	0.0205^{a}	0.0707^{a}	0.0554^{a}	0.0470^{a}	0.0023
Alga only	0.0000	-0.0002^{b}	0.0000^{b}	0.0002^{b}	0.0006^{b}	0.00224
LAB only	0.0006	0.0214^{a}	0.0714^{a}	0.0598^{a}	0.0569^{a}	0.00226
Algae + LAB	0.0000	0.0000^{b}	0.0000^{b}	0.0000^{b}	0.0003^{b}	0.00206
			CO_2 Fraction			
Control	0.94^{a}	0.78	0.8	0.74	0.69	0.064
Alga only	0.80^{bc}	0.78	0.75	0.8	0.74	0.0626
LAB only	0.90^{ab}	0.84	0.83	0.86	0.8	0.0643
Algae + LAB	0.77^{c}	0.83	0.75	0.81	0.67	0.0617

$^{a-b}$ Means within a column not connected by the same letter are significantly different ($P < 0.05$),

Table 2.3: The effect of *Asparogopsis taxiformis* and *Lactobacillus plantarum* on net production between time steps of H_2, CH_4 and CO_2 production at different time interval 0, 4, 24, 48 and 72 hours in vitro.

Treatment	0 HR	4 HR	24 HR	48 HR	72 HR	SEM
			Net H_2 (mmol)			
Control (A)	0	0.001[b]	-0.002[b]	0.001[b]	0.018	0.0165
Alga only (B)	0	0.123[a]	0.378[a]	0.149[a]	0.041	0.0157
LAB only (C)	0	0.002[b]	-0.001[b]	0.008[b]	0.005	0.0182
Algae + LAB (D)	0	0.129[a]	0.364[a]	0.132[a]	-0.011	0.0152
			Net CH_4 (mmol)			
Control (A)	0	0.1567[a]	0.4956[a]	-0.0525	-0.0441	0.02538
Alga only (B)	0	0.0004[b]	0.0000[b]	0.0000	0.0024	0.02465
LAB only (C)	0	0.1609[a]	0.5010[a]	-0.0488	0.0399	0.02583
Algae + LAB (D)	0	0.0000[b]	0.0000	0.0000	0.0019	0.02425
			Net CO_2 (mmol)			
Control (A)	0	1.01	2.71	0.05	-0.15	0.41298
Alga only (B)	0	1.42	2.4	0.92	0.34	0.39923
LAB only (C)	0	1.6	2.63	0.47	0.5	0.41935
Algae + LAB (D)	0	1.92	1.85	0.87	-0.51	0.39063

The net individual gas production mmol were derived using equation 3. Values indicate net change in gas between timesteps. Negative values indicate consumption of gas type.

[a-b]Means within a column not connected by the same letter are significantly different ($P < 0.05$).

Table 2.4: The effect of *Asparogopsis taxiformis*, *Lactobacillus plantarum* and their combination on cumulative H_2, CH_4 and CO_2 production at different time interval 0, 4, 24, 48 and 72 hours in vitro.

Treatment	0HR	4HR	24HR	48HR	72HR	SEM
			H_2 Cumulative (mmol)			
Control	0	0.0014[b]	-0.0004[b]	0.0024[b]	0.0189[b]	0.0277
Alga only	0	0.1233[a]	0.4895[a]	0.6299[a]	0.6315[a]	0.0269
LAB only	0	0.0020[b]	0.0014[b]	-0.0051[b]	0.0078[b]	0.0282
Algae + LAB	0	0.1288[a]	0.4930[a]	0.6248[a]	0.6143[a]	0.0266
			CH_4 Cumulative (mmol)			
Control	0	0.1567[a]	0.6515[a]	0.5728[a]	0.5360[a]	0.0181
Alga only	0	0.0004[b]	0.0000[b]	0.0018[b]	0.0035[b]	0.0173
LAB only	0	0.1609[a]	0.6619[a]	0.6215[a]	0.6103[a]	0.01892
Algae + LAB	0	0.0000[b]	0.0000[b]	0.0000[b]	0.0019[b]	0.0169
			CO_2 Cumulative (mmol)			
Control	0	1	3.8	3.4	3.3	0.57
Alga only	0	1.4	3.3	4	4	0.55
LAB only	0	1.6	4.2	4.6	4.3	0.57
Algae + LAB	0	1.9	3.8	4.6	4.1	0.54

[1]Cumulative individual gas production (mmol) was calculated from equation 4. Values indicate net change in gas between timesteps. Negative values indicate consumption of gas type.

[a-b]Means within a column not connected by the same letter are significantly different ($P < 0.05$).

Chapter 3: Effect of sodium sulfite on gas and volatile fatty acids parameters in vitro

ABSTRACT

Sulfite is used as a preservative to prevent food oxidation and can inhibit growth of some microorganisms. It may be present in some feed ingredients. However, limited research has investigated its effects on ruminal fermentation. The objective of study is to identify the safest level of sulfite inclusion in the rumen that would produce an effect on fermentation parameters. The hypothesis of the study was that sulfite inhibits methanogenesis, while increasing hydrogen and propionate concentration. Experimental design consisting of 4 treatment levels, 5 sampling times and 5 replicates for each level of treatment. The experiment was repeated twice (blocks). In the in vitro experiment, four levels of sodium sulfite (0, 0.1, 0.2, 0.4 mM) were tested in a randomized design with five replications per run (block) across two runs (blocks). A 1:1 mixture (50 ml) of rumen fluid from a non-lactating Holstein cow and in vitro medium were added to Wheaton bottles with timothy hay (0.75 g). Gas and VFA samples were analyzed by gas chromatography at 0, 4, 8, 24, and 48 hours. Data were analyzed using JMP® Student Edition 19.0.1. Total gas production and net methane were lower at the highest level of sulfite addition compared with the control at 4 h (164 vs. 180 mL; 0.05 vs. 0.23 mmol) and 8 h (145 vs. 159 mL; 0.18 vs. 0.39 mmol) of incubation. However, gas production became higher at 24 h (192 vs. 177 mL) and 48 h (134 vs. 128 mL; $P < 0.05$), suggesting compensation for the earlier inhibition. Net H_2 production was higher with sulfite than the control at 4 h (0.077 vs. 0.00245 mmol), whereas H_2 consumption was greater for the same treatments at 24 h ($P < 0.05$). Concentrations of acetate (54 vs. 64 mmol), propionate (45 vs. 47 mmol), butyrate (29 vs. 32 mmol), and total VFA (129 vs. 144 mmol) were lower with sulfite at 8 h but recovered thereafter ($P < 0.05$). The

acetate:propionate ratio remained lower at the highest sulfite concentration compared with the control from 4 to 48 h. Overall, addition of sulfite to in vitro batch cultures delayed ruminal fermentation but did not affect fermentation end products by 24 h.
Key words: hay, methane, gas, sulfite, VFA,

INTRODUCTION

Ruminants harbor quadrillions of microbes in their rumen, which are responsible for degrading feed and producing essential metabolites required for their daily functions. The rumen favors proliferation of anaerobic microorganisms. Feed digestion by these microbes results in the release of gases, volatile fatty acids, lactic acid, and ethanol (Cammack et al., 2018). Among the gases, carbon dioxide (CO_2), methane (CH_4), and hydrogen (H_2) are produced in varying amounts. Although H_2 is produced in smaller quantities, it plays a crucial role in maintaining fermentation and supporting ATP generation by several H_2-dependent microbes, including propionate producers and methanogens (Mackie et al., 2024). Amongst all, methanogens possess a high affinity for H_2, utilizing it to reduce CO_2 into methane (Fonty et al., 2007).

Methane is a potent greenhouse gas with a 100-year global warming potential (GWP_{100}) that is 28 times greater than that of CO_2 (Change, 1990) on a mass basis (USA-EPA, 2024). Enteric methane emissions from ruminants comprise about 2% of total anthropogenic greenhouse emissions. In addition to its environmental impact, methane production also represents an energy loss of up to 10% of the feed's metabolizable energy, thereby reducing overall feed efficiency (Johnson & Johnson', 1995). The growing global demand for ruminant products such as milk, meat, and fiber could further increase greenhouse gas emissions from ruminants (Kristiansen et al., 2021). At present, improving ruminant productivity while minimizing environmental impacts remains a major challenge, as most available methane mitigation strategies are costly and often fail to significantly enhance productivity.

3-nitrooxypropanol (3-NOP), sea weeds, and essential oils are effective in altering the fermentation pathways and reducing methane. Each of these inhibitors have different specific functions that interfere directly or indirectly with methanogens and eventually inhibits methane forming pathways. For example, 3-NOP reduced the methane emissions by 22-31% (g/day) (Kirwan et al., 2024; Lahart et al., 2025), whereas red algae reduced methane by 26% to 63% in vivo depending on the dose of algae (Colin et al., 2024). But 3-NOP at present requires incentive and governmental approval to be cost effective as a feed additive (Pupo et al., 2025) and production cost of the sea weeds in large scale is extremely high. Therefore, the discovery of new products or strategies to reduce methane emissions remains an area of great interest for researchers.

In this study, we investigated the effect of the inorganic salt sodium sulfite on rumen fermentation parameters. Sulfite is an intermediate product formed during the reduction of sulfate to hydrogen sulfide (Choudhury et al., 2022). In vivo and in vitro studies have reported that sulfite salts can reduce methane production while increasing the proportions of propionate and hydrogen in the rumen. Sodium sulfite has been suggested to exert a direct toxic effect on methanogens (Van'nevel et al., 1970) however, its exact mechanism of action has not yet been clearly elucidated. But sulfite can be degraded by certain microbes in the rumen, which could lead to the inefficiency of the sulfite compound over time. Therefore, the objective of this study was to determine the safest and most effective dose level of sodium sulfite to be included in the diet to obtain the desired fermentation results in both in vitro in the current and in vivo future studies.

MATERIALS AND METHODS

Rumen fluid collection and preparation

The experimental procedures were approved by the University of Maryland Institutional Animal Care and Use Committee (IACUC#- R-MAY-25-18) ahead of the starting date of experiment. Rumen fluid was collected from a non-lactating, fistulated cow at the college farm. Fifty-milliliter centrifuge tubes were used to withdraw both fluid and fibrous content from the rumen of the cannulated cow before the morning feeding. Since the farm is only a five-minute walk from the laboratory, the rumen fluid was processed immediately upon arrival. The fluid and fibrous content were homogenized using a blender for 20 seconds, then transferred to an Erlenmeyer flask under continuous CO_2 perfusion. The mixture was filtered through four layers of cheesecloth lined with glass wool to remove residual fiber. An in vitro reduced medium was prepared on the same day. Rumen fluid and the medium were combined in a 1:1 (v/v) ratio and mixed thoroughly using a magnetic stir bar.

Wheaton bottles (150 mL) were prepared one day prior to the experiment, with 0.75 g of ground Orchard hay added to each. The bottles were sealed with rubber stoppers and aluminum caps, then flushed with CO_2 using a 20-gauge inlet and a 22-gauge outlet needle for at least 25 minutes to ensure complete anaerobic conditions. A 60 mL syringe fitted with a 20-gauge needle was used to distribute 50 mL of the rumen fluid-medium mixture into each bottle. The mixture was randomly allocated among bottles according to the respective treatments. After transferring the fluid, excess gas inside the bottles was released using a 100 mL glass syringe to bring the internal pressure to approximately 1 atm.

Treatment

On the same day, a 0.5 M sodium sulfite stock solution was prepared. Different volumes of sodium sulfite solution (0, 0.1, 0.2, and 0.4 mL) were injected into appropriately labeled bottles to obtain final sulfite concentrations of 0, 0.1, 0.2, and 0.4 mM, respectively. The treatments were arranged in a randomized complete block design as a 4 × 5 factorial, with 4 sulfite levels (0, 0.1, 0.2, and 0.4 mM) and 5 repeated sampling times (0, 4, 8, 24, and 48 h). All bottles were incubated at 39 °C for 48 hours in an incubator placed on a shaker.

Gas Sampling

The first and subsequent gas were collected after 10 minutes of microbial adjustment and at 4, 8, 24, and 48 hours of incubation, respectively. A Fortuna glass syringe fitted with a 23-gauge needle was used to collect and measure the volume of gas. A 10 mL plastic syringe equipped with two 2-way Luer-Lock stopcock valves was used to collect gas samples from each bottle. Similarly, a 3 mL syringe with a 23-gauge needle was used to collect VFA samples (1-1.5 mL), which were then transferred into 2 mL centrifuge tubes and immediately stored at -20 °C for later analysis. Gas and VFA samples were subsequently analyzed using gas chromatography (GC).

Gas analysis on GC

A 1 mL Pressure-Lok® precision analytical syringe was used to inject gas samples into the GC inlet. Before injection, the syringe was flushed at least three times with the respective fermented gas to avoid air contamination. On the day of VFA analysis, the sample tubes were removed from the freezer and allowed to thaw. After

thawing, the tubes were centrifuged at 7,000 × g for 15 minutes. A 0.7 mL aliquot of the supernatant was transferred into GC vials, mixed with 0.3 mL of 10% phosphoric acid (H_3PO_4, v/v), and sealed with aluminum caps and mixed well by inverting vials couple times. Gas samples were analyzed using a glass-packed column, whereas VFA samples were analyzed using capillary column in Gas Chromatography. Data were extracted from GC files into excel and analyzed using JMP® Student Edition 19.0.1.

Gas equations

Total gas production, cumulative gas production, net individual gas production, change in individual gas production, cumulative, rate of individual gas formation were calculated based on the following equation:

$$V_t = V_{s,t} + V_h \quad \ldots\ldots\{1\}$$

$$V_{cum,t} = V_h + \sum_{t=0}^{t} V_{s,t} \quad \ldots\ldots\ldots\{2\}$$

$$X_t = \frac{(V_{s,t} \times C_t) + V_h (C_t - C_{t-1})}{22.4} \quad \ldots\ldots\ldots\{3\}$$

$$X_{cum,t} = X_t + \sum_{t=0}^{t} (X_{t-1}) \quad \ldots\ldots\ldots\{4\}$$

$$\frac{}{22.4}$$

Where,

V_t = volume of gas (mL) at time t

$V_{cum,t}$ = Cumulative gas production at time t (mL)

X_t = Net production of individual gas (CH_4, H_2 and CO_2) at time point (t) (mmol)

$X_{cum,t}$ = Cumulative individual gas production at time t (mmol)

$V_{s,t}$ = Volume of gas collected in syringe at time t, and

V_h = Volume of headspace

Vs_{t-1} = Volume (mL) of gas in syringes in previous time point

C_t = Concentration of individual gas at time t and 22.4 is the molar volume of an ideal gas at STP.

C_{t-1} = Concentration of individual gas in previous time point (t-1)

X_{t-1} = Production of individual gas (CH_4, H_2 and CO_2) at time point (t-1) (mmol)

Statistical analysis

The statistical model included 4 levels of treatment, and 5 different time points. Data was analyzed using one-way Anova, the comparison were made between the treatment groups within each time point. Batch was included in the model as a random effect. As the analysis was done for individual time points, the variable 'time' was used as a by variable in the JMP software, with a separate analysis done for each individual time point (0, 4, 8, 24, and 48 hr).

The statistical model was:

$$Y_{bijk} = \mu + B_b + T_i + R_j + \varepsilon_{bijk}$$

Where,

Y_{bijk} = observed response (e.g., CH_4, H_2, or VFA concentration),

μ = overall mean,

B_b = random effect of batch,

T_i = fixed effect of treatment,

R_j = random effect of batch

ε_{bijk} = residual error.

All analyses were performed in JMP® Student Edition 19.0. and significance was declared at $P < 0.05$ and tendency towards statistical differences was declared at $0.05 < P < 0.10$.

RESULTS

Total gas

The total gas production at 4 hours were 180, 176, 172, and 164 mL for 0, 0.1, 0.2, and 0.4 mM, respectively, indicating reduced gas production at the highest inclusion level (Table 3.1). A similar pattern was observed at 8 hours, where production was 159, 162, 159, and 145 mL, respectively. However, at 24 hours and 48 hours the gas production improved in 0.4 mM sulfite, with total gas production increasing to 177, 178, 181, and 192 mL at 24 hours, whereas at 48 hours to 128, 130, 131, and 134 mL for 0, 0.1, 0.2, and 0.4 mM, respectively (Table 3.1).

The cumulative gas production at 4 hours were 180, 176, 172, and 164 mL for 0, 0.1, 0.2, and 0.4 mM, respectively, and at 8 h were 224, 223, 221, and 197 mL, indicating lower gas accumulation in the 0.4 mM treatment during early incubation. At 24 h, cumulative gas production was 284, 286, 288, and 269 mL, whereas at 48 h they were 299, 301, 303, and 290 mL for 0, 0.1, 0.2, and 0.4 mM, respectively (Table 3.1).

Hydrogen

Hydrogen concentration and cumulative production were higher in the 0.4 mM sulfite treatment than in all other doses, and this effect lasted for up to 48 hours ($P < 0.05$). No difference was observed for the 0.1- and 0.2-mM treatments compared with the control (Table 3.3). Additionally, net hydrogen production was higher (0.07796 vs

0.00245, 0.00150, 0.00256 mmol), in 0.4 mM sulfite inclusion at 4-h timepoint, where less (-0.04620 vs -0.00073, -0.00082, -0.00112 mmol) amount being produced at time point 24 hours than rest of the treatments (Table 3.3).

Methane

Methane concentration and cumulative net methane production were lowest in the 0.4 mM sulfite treatment compared with the other doses starting at 4 to 24 hours (Table 3.2 & Table 3.3). Net methane production was affected by sodium sulfite during early incubation. At 4 hours, the net CH_4 production were 0.23, 0.20, 0.16, and 0.05 mmol for 0, 0.1, 0.2, and 0.4 mM, respectively, indicating that sulfite had a dose-dependent effect on methane reduction, with the greatest inhibition observed at 0.4 mM. At 8 hours, the sulfite effect persisted, with net CH_4 production averaging 0.39, 0.31, 0.34, and 0.18 mmol for the respective treatments. In contrast, treatment effects were not apparent at 24 and 48 h, when methane production was similar among treatments. (Table 3.3).

Carbon dioxide

The net carbon dioxide production (mmol) was highest in control than 0.4 mM concentration of the sulfite inclusion at time point 4 hours only (Table 3.3). No effect of treatment on rest of time points, however, cumulative CO_2 was only differed at time 48 hours where 0.4 mM sulfite culture had the most CO_2 (6.8 mmol) comparted to control (5.6 mmol) and 0.1 mM sulfite (5.6 mmol) (Table 3.4).

Volatile Fatty Acid Parameters

There was a trend for a treatment effect on acetate concentration at time 4 h. Acetate was lower at the highest sulfite level (0.4 mM) at 4, 8, and 24 h compared with 0,

0.1, and 0.2 mM of sulfite levels. No differences among treatments were found at 0 h or 48 h (Table 3.5). For propionate, the treatment effect was significant at 8 h only, the 0.4 mM sulfite treatment resulted 45 mM versus 47 mM at 0, 0.1, and 0.2 mM of propionate concentration. No differences were observed at other time points. Similarly, on butyrate, the treatment effect was only at 8 h, where 0.4 mM had the lowest concentration (29.5 mM) compared with 32.7, 31.8, and 31.3 mM for 0, 0.1, and 0.2 mM sulfite levels, respectively (Table 3.5). At 8 h, total VFA concentration was higher in the 0-, 0.1-, and 0.2-mM sulfite treatments than in 0.4 mM. At 0, 4, 24, and 48 h, there was no dose effect on total VFA across sulfite levels (Table 3.5). The acetate: propionate (A:P) ratio was lowest at 0.4 mM compared with the other doses, except at 0 h, where no difference was detected (Table 3.5).

DISCUSSION

Gas production

The effect of higher dose of sulfite was evident at time point 4 and 8 hours where, total gas and cumulative production were 9% and 12% lower, respectively. And total gas production then started increasing until 48 hours for the 0.4 mM sulfite treatment compared with the other doses. No effect on cumulative gas production was seen after 8 hours of incubation. These results align with previous findings that increasing the dose of sodium sulfite decreased the methane production when CO_2:H_2 and formate were used as substrate in vitro (Van Nevel et al., 1970). The reduction in biogas production can result from the increasing concentration of H_2 at time point 4 and 8 hours in 0.4 mM of sulfite inclusion. During fermentation, H_2 production is a key electron sink where reducing equivalents (e.g., NADH) are re-oxidized to NAD^+ (Ungerfeld, 2020). This re-oxidation

is essential for the continued degradation of organic matter by microorganisms in an anaerobic environment.

A high partial pressure of H_2 can thermodynamically inhibit hydrogenase activity, resulting in limited reoxidation of NADH to NAD^+ (Mackie et al., 2024). This decreases glycolytic flux and substrate degradation, eventually lowering total biogas production (Ungerfeld, 2015). The organic matter degradation seemed improved after accumulated hydrogen being used up by micro-organisms in later half of the study and maintained the optimum H_2 concentration. Because total gas production was improved in the highest concentration of the sodium sulfite inclusion at 24 and 48 hours. With the decrease in H_2 concentration at 24 and 48 hours, the feedback inhibition appeared to be reversed, allowing greater fermentation of feed and fiber indicate greater H_2 consumption and reduced production at the 8- and 24-hour time points in the 0.4 mM sulfite treatment compared with the other treatments. This effect is likely due to reduced total gas production caused by feedback inhibition from accumulated hydrogen gas (Ungerfeld, 2015). In contrast, H_2 production reversed at 48 hours in the 0.4 mM treatment, where the rate of reaction increased significantly compared with the other treatments. This increase was mostly caused decreased total gas production at this time point. The high cumulative H_2 production in the 0.4 mM treatment throughout the experiment suggests that the compound has the capacity to enhance H_2 production, but the reaction appears to be dose dependent.

Lower methane production was expected with higher doses of sodium sulfite. In the current study, the effect of sodium sulfite was pronounced in the highest dose of its inclusion and overall methane production (cumulative) was suppressed up to 24 hours

only in 0.4 mM level of sodium sulfite. A similar in vitro study using CO_2: H_2 and formate as substrates for methanogenesis, with sodium sulfite as an inhibitor, also reported a significant reduction in methane production in both treatments. An in vivo study further demonstrated that methane production decreased when wethers were infused with 40 mL of a 10% Na_2SO_3 (w/v) solution through the fistula (equivalent to 8 g sulfite/day) (Van Nevel et al., 1970). Notably, the methane production at the 4-hour time point indicates that the efficacy of sodium sulfite was highest during the early stages of fermentation, even at the 0.2 mM level, compared with the 0- and 0.1-mM treatments.

However, as fermentation progressed, the efficacy of the inhibitor appeared to decline. As a result, no differences in net methane production were observed after 8 hours, even at the 0.4 mM sulfite inclusion level. The reduced inhibition may be due to rapid microbial degradation of the compound, which likely diminished its effectiveness mostly after 8 hours and at later time points. The inefficiency of sulfite in reducing methane at lower doses during later time points supports previous findings that some species of methanogens grow optimally at sulfite concentrations even higher than our dose that is 0.5 mM to 1 mM (Daniels et al., 1986). Because some methanogens have been reported to detoxify sulfite through an F_{420}-dependent sulfite reductase pathway reducing sulfite to sulfide as they may utilized sulfite as an inorganic sulfur source for their own growth.

Although detailed information on the mechanism by which sulfite decreases methane is limited, early researchers speculated that sulfite exerts direct toxicity toward methanogens, and its inclusion in fermentation cultures consequently suppresses methane production (Van Nevel et al., 1970). Therefore, the no difference on methane formation

observed at the 48-hour time point between treatments couldd be due to the loss of inhibitory effects of it combined with an enhanced glycolytic flux resulting from increased re-oxidation of reducing equivalents.

The lower net CO_2 production at the highest level of sulfite addition may be attributed to reduced total gas output. Additionally, during methanogen inhibition, CO_2-utilizing pathways may shift toward reductive acetogenesis instead methanogenesis (Ungerfeld, 2015). However, when methanogenesis is inhibited and H_2 accumulates, the thermodynamic environment becomes more favorable for acetogens to use CO_2 and H_2 to produce acetate, resulting in reduced net CO_2 release (Kohn & Boston, 2000). In contrast, under normal conditions, acetogens are suppressed because methanogens outcompete them by scavenging nearly all available hydrogen (Ungerfeld, 2015).

Volatile Fatty Acids

Acetate concentration was lowest in the highest level of sulfite infusion compared to the control at the 4-hour time point. No such effect was observed at the 0.1 mM and 0.2 mM levels of sulfite on acetate production relative to the control. An in vitro study also reported a similar trend, where acetate production decreased (1817, 1594, and 1461 μmol/vessel) with increasing levels of sulfite inclusion ($0 < 0.1 < 0.2$ mM) when glucose was used as the substrate (Van Nevel et al., 1970). Similarly, sulfate decreased methane production by 16% in growing male lamb when added in the diet (van Zijderveld et al., 2010) . In agreement, the increased H_2 concentration under methane-inhibited conditions negatively affected acetate production and redirected H_2 toward alternative electron sinks (Janssen, 2010) Because, acetate is produced through oxidative fermentation, during which substrate oxidation releases reducing equivalents that are taken up by protons to

form H_2. This pathway is favorable only when the partial pressure of H_2 (pH_2) is low, resulting in a strongly negative Gibbs free energy change (Ungerfeld, 2020). However, the inhibitory effect of sulfite on acetate production was not observed at the 48-hour time point.

Theoretically, an increased level of pH_2 decreased the acetate production would favor propionate pathway; however, in this study, propionate production showed the opposite trend. The concentration of propionate was reduced in the 0.4 mM sulfite treatment compared to the control, 0.1 mM, and 0.2 mM treatments at the 8-hour time point. This result aligns with the findings of Van Nevel et al. (1970), who reported that the inclusion of sodium sulfite in a rumen culture containing glucose as the substrate decreased propionate production by 42% at 0.2 mM sulfite. However, the same researchers observed a 25% increase in propionate production in an in vivo study where wethers were infused with 8 grams of sulfite per day. This could be attributed to the activation of alternative hydrogen-utilizing pathways, such as those involving sulfate-reducing bacteria, which compete with propionate formation for available reducing equivalents. Sulfite serves as a key intermediate in both assimilatory and dissimilatory sulfate reduction pathways, where it acts as an electron acceptor and is ultimately reduced to sulfide as the end product (Kushkevych, Cejnar, et al., 2020). However, the trend at the 24- and 48-hour time points showed numerically higher concentrations of propionate in the 0.4 mM sulfite treatment compared to the control. This may have occurred due to the accumulation of H_2S, which could have eventually suppressed the sulfate reduction pathways, thereby redirecting reducing equivalents toward propionate formation.

Like propionate, a similar trend of butyrate concentration was seen in this study, where butyrate concentration was low at timepoint 8 hours in 0.4 mM level of sulfite inclusion vs rest of other dose responses. This finding aligns with a previous study conducted using rumen ingesta containing hay, where the inclusion of sodium sulfite increased butyrate concentration from 0.45 to 0.72 meq acid/100 mL (0 vs. 75 mg sodium sulfite/100 mL ingesta) during 8-hour incubation period (Krabill et al., 1969a). Butyrate producers are typically hydrogen (H_2) producers at low pH_2 (Qu et al., 2022). In a syntrophic association with methanogens, the H_2 produced from butyrate formation is captured by methanogens, which maintain pH_2 and redox balance, this interspecies hydrogen transfer provides thermodynamic favorability for butyrate producers (Cong et al., 2021), allowing the reaction to proceed towards more butyrate formation. But under methane-inhibitory and high pH_2 conditions, where interspecies H_2 transfer is blocked, the elevated H_2 concentration inhibits electron flow through the hydrogenase enzyme, resulting in thermodynamic unfavourability and reduced butyrate production.

A:P ratio and Total VFA

In this study, the total VFA concentration was lower in the high-dose treatment, particularly at the 8-hour time point than control. This could be because the effect of sodium sulfite was more pronounced in reducing acetate than propionate. Van Nevel et al. (1970) reported a decline in total VFA production with increasing doses ($0 < 0.1 < 0.2$ mM) of sulfite addition, where total VFA concentrations decreased from 2950 to 2572 and 2341 μmol/vessel in rumen liquor incubated with glucose as a substrate, respectively. Since acetate constitutes the majority of total VFA, a decline in production due to excess H_2 accumulation would substantially reduce the overall VFA concentration. The A:P

ratio decreased in the 0.4 mM sodium sulfite treatment compared with the control and the other sulfite inclusion levels. Although both acetate and propionate concentrations were reduced at the highest dose, the greater decline in acetate suggests that the treatment effect was more pronounced on acetate production than on propionate. The reduction in total VFA concentration further indicates that the lower A:P ratio resulted from decreased production of individual VFAs rather than a shift in fermentation toward propionate.

Summary

Collectively, this study demonstrated that the addition of sodium sulfite was effective in reducing methane production; however, the response was dose dependent. The effect of the treatment on methane reduction and H_2 accumulation appeared immediately but was sustained only at the highest dose of sodium sulfite. Similarly, both individual and total VFAs were influenced by the higher levels of treatment inclusion, with most of the effects observed at the 8-hour time point. Overall, this study indicates that the effect of sodium sulfite was temporary because microbes seemed to be adapted or catabolize the added sulfite. Further research is warranted to validate these findings under in vivo conditions and to translate the in vitro results into actual animal performance outcomes.

Table 3. 1: The effect of different levels of sodium sulfite on total gas (mL) at 1 atm. measured at each time step (gas in excess of bottle headspace volume was discarded after measurement) and cumulative gas (mL) production at 0, 4, 8, 24, and 48 hours of time interval in vitro.

			Total gas mL			
Treatment	**0HR**	**4HR**	**8HR**	**24HR**	**48HR**	**SEM**
0 mM	115	180[a]	159[a]	177[b]	128[b]	4.2
0.1 mM	115	176[ab]	162[a]	178[b]	130[b]	4.2
0.2 mM	115	172[bc]	159[a]	181[b]	131[ab]	4.2
0.4 mM	115	164[c]	145[b]	192[a]	134[a]	4.2
			Cumulative gas mL			
0 mM	115	180[a]	224[a]	284	299	6.7
0.1 mM	115	176[ab]	223[a]	286	301	6.8
0.2 mM	115	172[bc]	221[a]	288	303	7.1
0.4 mM	115	164[c]	197[b]	269	290	6.8

The total gas (mL) and cumulative gas (mL) were derived using equation 1 and 2.

[a-c] Means within a row not connected by the same letter are significantly different ($P < 0.05$).

Table 3.2: The effect of different levels of sodium sulfite on concentration of H_2, CH_4 and CO_2 at 0, 4, 8, 24, and 48 hours of time interval.

H_2 concentration

Treatment	0 HR	4 HR	8 HR	24 HR	48 HR	SEM
0 mM	0.00025[b]	0.00046[b]	0.00036[b]	0.00010[b]	0.00013[b]	0.0003102
0.1 mM	0.00037[b]	0.00043[b]	0.00032[b]	0.00010[b]	0.00013[b]	0.0003102
0.2 mM	0.00057[a]	0.00071[b]	0.00038[b]	0.00011[b]	0.00017[ab]	0.0003104
0.4 mM	0.00065[a]	0.01112[a]	0.00778[a]	0.00015[a]	0.00020[a]	0.0003102
			CH_4 concentration			
0 mM	0.0028[a]	0.0297[a]	0.0738[a]	0.1131[a]	0.1122	0.00448
0.1 mM	0.0021[ab]	0.0270[a]	0.0616[a]	0.1095[a]	0.1053	0.00444
0.2 mM	0.0017[b]	0.0214[b]	0.0637[a]	0.1084[a]	0.108	0.00458
0.4 mM	0.0014[b]	0.0075[c]	0.0337[b]	0.0906[b]	0.0974	0.00456
			CO_2 concentration			
0 mM	0.857	0.807	0.698	0.684	0.678	0.11864
0.1 mM	0.833	0.792	0.606	0.68	0.638	0.11838
0.2 mM	0.842	0.755	0.687	0.689	0.681	0.1185
0.4 mM	0.827	0.76	0.721	0.652	0.688	0.11874

[a-c] Means within a row not connected by the same letter are significantly different ($P < 0.05$)

Table 3.3:The effect of different levels of sodium sulfite on net production between timesteps of H_2, CH_4 and CO_2 at 0, 4, 8, 24, and 48 hours of time interval.

			Net H_2 mmol			
Treatment	**0 HR**	**4 HR**	**8 HR**	**24 HR**	**48 HR**	**SEM**
0 mM	0	0.00245[b]	0.0004	-0.00073[a]	0.00030	0.003375
0.1 mM	0	0.00150[b]	0.00008	-0.00082[a]	0.00022	0.003325
0.2 mM	0	0.00256[b]	-0.00097	-0.00112[a]	0.00042	0.003325
0.4 mM	0	0.07796[a]	-0.00537	-0.04620[b]	0.00034	0.003375
			Net CH_4 mmol			
0 mM	0	0.23[a]	0.39[a]	0.52	0.11	0.047
0.1 mM	0	0.20[a]	0.31[a]	0.55	0.05	0.046
0.2 mM	0	0.16[b]	0.34[a]	0.54	0.1	0.047
0.4 mM	0	0.05[c]	0.18[b]	0.62	0.14	0.047
			Net CO_2 mmol			
0 mM	0	2.4[a]	1	1.8	0.7	0.40
0.1 mM	0	2.0[ab]	0.3	2.3	0.2	0.39
0.2 mM	0	1.6[b]	1	2	0.5	0.40
0.4 mM	0	1.3[b]	0.8	1.7	0.5	0.40

The net individual gas production mmol were derived using equation 3. The negative value indicates the consumption of gas.

[a-c] Means within a row not connected by the same letter are significantly different ($P < 0.05$).

Table 3.4: The effect of different levels of sodium sulfite on cumulative gas production at 0, 4, 8, 24, and 48 hours of time interval.

			H_2 mmol Cumulative			
Treatment	**0 HR**	**4 HR**	**8 HR**	**24 HR**	**48 HR**	SEM
0 mM	0.0013[b]	0.0037[b]	0.0038[b]	0.0014[b]	0.0010[a]	0.0025
0.1 mM	0.0019[b]	0.0034[b]	0.0035[b]	0.0015[b]	0.0010[b]	0.0025
0.2 mM	0.0029[a]	0.0055[b]	0.0045[b]	0.0016[b]	0.0013[b]	0.00258
0.4 mM	0.0033[a]	0.0813[a]	0.0753[a]	0.0141[a]	0.0017[a]	0.00258
			CH_4 mmol Cumulative			
0 mM	0.014[a]	0.240[a]	0.628[a]	1.038[a]	0.927	0.05365
0.1 mM	0.011[ab]	0.213[a]	0.521[a]	0.997[a]	0.915	0.0539
0.2 mM	0.009[b]	0.168[b]	0.511[a]	1.000[a]	0.967	0.0553
0.4 mM	0.007[b]	0.055[c]	0.238[b]	0.819[b]	0.935	0.05401
			CO_2 mmol Cumulative			
0 mM	4.4	6.5	7.5	6.8	5.6b	1.1
0.1 mM	4.3	6.2	6.6	6.7	5.6b	1.1
0.2 mM	4.3	5.9	7	6.9	5.8ab	1.1
0.4 mM	4.2	5.6	6.4	6.6	6.8a	1.1

Cumulative individual gas production (mmol) was calculated from equation 4.

[a-c] Means within a row not connected by the same letter are significantly different ($P < 0.05$).

Table 3.5: Least square mean concentration of acetate, propionate, butyrate, total VFA and A:P ratio at different levels of sodium sulfite inclusion across 0, 4, 8, 24, and 48-hour time points.

Treatment	0HR	4HR	8HR	24HR	48HR	SEM
			Acetate mM			
0 mM	38	47[a]	64[a]	77[a]	81	2.6
0.1 mM	37	44[ab]	63[a]	77[a]	80	2.6
0.2 mM	37	45[ab]	62[a]	77[a]	84	2.6
0.4 mM	37	43[b]	54[b]	70[b]	82	2.6
			Propionate mM			
0 mM	15	28	35[a]	41	43	2.1
0.1 mM	13	26	34[a]	41	43	2.1
0.2 mM	14	28	34[ab]	42	45	2.1
0.4 mM	15	28	33[b]	40	45	2.1
			Butyrate mM			
0 mM	14	20	32[a]	42	44	4.4
0.1 mM	14	18	31[a]	42	44	4.4
0.2 mM	14	19	31[a]	43	47	4.4
0.4 mM	15	19	29[b]	41	48	4.4
			Total VFA mM			
0 mM	73	96	132[a]	160	168	7.6
0.1 mM	70	88	130[a]	162	169	7.6
0.2 mM	71	93	127[a]	162	176	7.7
0.4 mM	73	90	117[b]	152	176	7.6
			Acetate:Propionate			
0 mM	1.9	1.7[a]	1.9[ab]	1.9[a]	1.9[a]	0.06
0.1 mM	1.9	1.7[ab]	1.9[a]	1.9[a]	1.9[a]	0.06
0.2 mM	1.9	1.6[bc]	1.8[b]	1.9[a]	1.9[a]	0.06
0.4 mM	1.9	1.6[c]	1.7[c]	1.7[b]	1.8[b]	0.06

[a-c] Means within a row not connected by the same letter are significantly different ($P < 0.05$).

Chapter 4: Effect of sodium sulfite or sodium acetate on feed intake and milk production in dairy cattle

ABSTRACT

Sodium sulfite and sodium acetate are commonly used food preservatives that can also influence rumen fermentation when included in ruminant diets. Sodium sulfite has been reported to decrease acetate to propionate molar ratio, whereas acetate serves as a key precursor for milk fat synthesis. Additionally, no one has measured effects of sulfite on methane emissions in dairy cattle. However, few studies have evaluated their roles in feed efficiency or lactational performance. Therefore, the objective of this study is to evaluate the effects of dietary sodium sulfite and sodium acetate on animal performance, dry matter intake, body weight and milk production. It was hypothesized that sodium sulfite will increase milk yield by increasing the availability of the energy precursor propionate, whereas dietary sodium acetate will enhance milk fat percentage. 32 multiparous Holstein cows (174 ± 41 days in milk; 40 ± 6 kg milk/d) were used in a replicated 4 × 4 Latin square design with 8 squares and four 21-d periods. Four original dietary groups were: bicarbonate, bicarbonate + sulfite, acetate, and acetate + sulfite. One square was comprised of 4 rumen fistulated cows, and 2 squares (8 cows) received a double dose of sulfite when assigned a treatment containing sulfite, resulting in 3 sulfite levels (0, 0.45, and 0.9% of DM) with or without 1% acetate in corn based TMR diet. Dry matter intake and milk yield were recorded daily, whereas body weight and body condition score were measured on day 18 of each period. Milk samples for composition analysis were collected during both the morning and evening milking on days 14 and 15 of each period.

Data were analyzed in the mixed model (JMP 19.0.1). Cows with 0.45% sulfite had greater body weight (690 kg) vs 0% (674 kg) or 0.9% sulfite (676 kg; SE = 12.0; P <

0.05). Milk yield decreased ($P < 0.05$) with 0.9% sulfite (37.1 kg/d) vs control (39.3 kg/d) and 0.45% (38 kg/d, SE = 1.07). Dry matter intake was higher in 0 than 0.45 and 0.9% sulfite levels (29.1, 27.9, and 27.8 kg/d; SE = 0.79; $P < 0.05$). Acetate did not affect milk yield, DMI, ECM, milk fat %, protein %, or fatty acid % ($P > 0.05$). Cows on 0.45% sulfite consumed less feed compared to those on 0 and 0.9% sulfite (27.1 vs. 28.0-28.4 kg/day). For the 3rd week, 0% sulfite diet had 30.9 kg/day DMI compared to 27.4 kg/day in 0.9% sulfite diet ($P < 0.05$). Milk composition was unchanged by sulfite and acetate diets, whereas period effects were observed for fat percentage, true protein percentage, urea concentration, and fatty acid profile ($P < 0.05$). Contrary to our hypothesis, sodium sulfite supplementation decreased milk yield, while sodium acetate had no effect on milk fat. Further analysis of VFA and gas parameters under similar dietary conditions may help explain these results.

Keywords: Fermentation, Lactating cattle, Milk, Sulfite,

INTRODUCTION

Ruminal fermentation in dairy cattle produces key metabolites, mainly the volatile fatty acids (VFA) acetate, propionate, and butyrate, which together account for about 95% of total ruminal VFA and can provide up to 70% of the metabolizable energy required for maintenance and production (Seymour et al., 2005). The typical molar proportions of these three major VFA in the rumen range from 63 to 67% acetate, 21 to 22% propionate, and 10 to 12% butyrate, depending on diet and physiological state (Nozière et al., 2011). Animal performance strongly depends on these proportions because acetate is the main precursor for milk fat synthesis, propionate is the primary gluconeogenic substrate supporting lactose yield, and butyrate is an important energy source for rumen epithelium and peripheral tissues (N. L. Urrutia & Harvatine, 2017). Consequently, even small alterations in microbial fermentation can shift the concentrations and proportions of major metabolites and reactive intermediates, with potentially large impacts on performance outcomes such as milk yield, body weight change, and body condition score. Therefore, the addition of these metabolites as feed additives is major interest of research at present.

Another metabolite that is formed during the reduction of sulfate to sulfide by sulfate reducing bacteria is a sulfite (Kushkevych et al., 2020). Although sulfite occurs in small fractions in the rumen, previous reports have shown that the inclusion of sulfite in the diet can affect the pattern of fermentation and the fate of total and individual VFA with concomitant reduction in methane emission (Van'nevel et al., 1970). There was only one study conducted on lactating dairy cows to observe the effect of 0.9% of DM (160 grams) sodium sulfite on animal productivity and rumen characteristics (Alhassan et al.,

1969). Whereas in vitro results suggested that a lower dose may be sufficient, the present study used two levels of sodium sulfite.

In addition, sodium acetate was used as an additive to serve as a precursor for milk fat synthesis; however, very few studies were conducted to observe the effects of sodium acetate on performance along with rumen fermentation characteristics. Thus, measuring the production characteristics such as dry matter intake, milk production, energy-corrected milk production, body weight, and milk composition would provide the most direct approach to observe the effects of the dietary treatment.

Therefore, the objective of the present study was to evaluate the effect of the sodium sulfite and sodium acetate on the animal production response of the lactating Holstein cow, with emphasis on the intake, milk yield, milk composition, and body weight. The hypotheses of the present study are that the increase of the sulfite compound would increase the DMI and milk production response of the cow, especially at the higher dose level, whereas the sodium acetate would have minimum effect on the production performance of the cow except for the milk fat.

MATERIALS AND METHOD

Animal, housing and feeding

The experimental procedures were approved by the University of Maryland Institutional Animal Care and Use Committee (IACUC#- R-MAY-25-18) ahead of the starting date of experiment. Thirty-two lactating multiparous Holstein-Friesian cows (n = 32; 174 ± 41 days in milk; 40 ± 6 kg milk/d), including four rumen-cannulated cows, were housed in a bedded-pack barn equipped with individual Calan gates at the Central Maryland Research and Education Center (CMREC), University of Maryland, College

Park. At the start of the trial, cows were in early to mid-lactation, and by the end of the experiment they had progressed to late lactation.

Cows were allocated to four treatment groups: bicarbonate, bicarbonate + sulfite, acetate, and acetate + sulfite (n = 8 per group). Within each group, six blocks of four cows received the regular treatment, whereas two blocks received a higher sulfite dose during each period, resulting in three sulfite levels (0, 0.45, and 0.9% of DM) without or with acetate (1% of DM). Two cows in different squares receiving 0.45% sulfite were removed from the study due to illness after the second experimental period.

Two blocks of four cows received 0.9% sulfite in combination with either bicarbonate or acetate, whereas the remaining blocks received the regular treatments containing bicarbonate or acetate without the higher sulfite dose. Treatment assignments were systematically rotated across periods to ensure balanced representation of treatments over time, resulting in a modified 4 × 4 Latin square experimental design, with 21-day experimental periods. Throughout the experimental periods, all cows were fed a basal diet formulated as a total mixed ration (TMR; Table 4.1). To minimize potential carryover effects between periods, treatment assignments were rotated such that each cow received different dietary treatments in successive periods, following a balanced treatment sequence across periods.

Prior to the start of Period 1, all experimental animals were fed the basal TMR for a three-week adaptation period. Diets were formulated to meet or exceed the nutrient requirements of lactating dairy cows according to the National Research Council (NRC, 2003).

Feed intake, body weight and body condition scores

Individual feed intake was recorded daily to calculate dry matter intake (DMI). The feed amount for each day was adjusted based on the previous day's refusals, with an additional 10% allowance. Refusals were collected early in the morning at 5300 h from each tub and weighed using a Data Ranger feed mixer. Fresh feed was then provided once a daily at 8300 h. Feed additives were prepared in advance and mixed into the TMR using the Data Ranger.

Body weight and body condition scores (BCS) of the cows were recorded on day 0, a day before the start of period 1, and again on the 17th day of each period. Measurements were taken early in the morning, immediately after milking. Body weight was recorded using a scale attached to the cow restraining area. BCS was evaluated by two different individuals to minimize bias, using the five-point (Elanco) scoring system.

Silage sampling and DM intake

Initially, only corn silage samples were collected twice weekly and dried using a Koster tester to monitor changes in dry matter (DM) concentration over time. During Period 4, following the onset of rainfall, entire TMR samples were collected daily, weighed, and dried overnight in a forced-air oven at 100 °C to determine DM percentage. In addition, a corn silage sample was collected from the bunker silo during the final week of each period and submitted to Cumberland Valley Analytical Services for forage quality analysis.

Because no substantial changes in corn silage DM concentration were observed across periods, DM intake was calculated using the initially determined TMR DM

percentage for Periods 1 through 3. For Period 4, DM intake was calculated using DM values determined by the overnight oven-drying method.

Milk samples

Cows were milked twice daily 7300 hr and 3300 hr. Milk samples from individual animals were collected on days 14 and 15 of each period. Milk samples were collected in the tubes containing preservatives and then mixed it thoroughly before poured 30-50 mL of tubes containing EDTA. Samples were kept at 4°C until shipped overnight to Lancaster DHIA, Pennsylvania, USA for analysis of fat, protein, lactose, MUN, and fatty acid profile.

Statistical Method

Body weight, body condition score (BCS), milk components, and energy-corrected milk (ECM) were analyzed using a mixed-effects model in ® JMP Student Edition 19.0.1 (SAS Institute Inc., Cary, NC, USA). The model included sulfite level, acetate level, the sulfite × acetate interaction, and period as fixed effects. Baseline milk yield and days in milk (DIM) were initially included as covariates to account for variation in pre-treatment milk production and stage of lactation. Cow was included as a random effect to account for repeated observations within animal across experimental periods.

The model was:

$$Y_{ijkl} = \mu + S_i + A_j + (S \times A)_{ij} + P_k + \beta_1 DIM_{ijkl} + \beta_2 BMY_{ijkl} + C_l + \varepsilon_{ijkl}$$

where Y_{ijkl} is the observed response variable; $\mu$is the overall mean; $S_i$is the fixed effect of sulfite level; A_j is the fixed effect of acetate level; $(S\times A)_{ij}$ is the interaction between

sulfite and acetate; P_k is the fixed effect of period; β_1 and β_2 are the regression coefficients for DIM and baseline milk yield, respectively; C_l is the random effect of cow; and ε_{ijkl} is the residual error.

Daily milk yield, daily feed intake, and dry matter intake (DMI) were analyzed using a mixed-effects model in JMP. Before finalizing the model, baseline milk yield and DIM were each evaluated separately as covariates in the presence of the other fixed effects. Because baseline milk yield significantly explained variation in the response variables whereas DIM did not, baseline milk yield was retained in the final model. The final model included sulfite level, acetate level, period, week, sulfite × week, acetate × week, centered baseline milk yield, sulfite × centered baseline milk yield, and acetate × centered baseline milk yield as fixed effects. Cow was included as a random effect, and repeated measurements over week were accounted for within cow-periods.

The model was:

$$Y_{ijklm} = \mu + S_i + A_j + P_k + W_l + (S \times W)_{il} + (A \times W)_{jl} + \beta CBMY_{ijklm} + (S \times CBMY)_i + (A \times CBMY)_j + C_m + \varepsilon_{ijklm}$$

Where, Y_{ijklm} is the observed response variable; $\mu$is the overall mean; S_i is the fixed effect of sulfite level; A_j is the fixed effect of acetate level; P_k is the fixed effect of period; W_l is the fixed effect of week; $(S\times W)_{il}$ and $(A\times W)_{jl}$ are the interactions of sulfite and acetate with week, respectively; β is the regression coefficient for centered baseline milk yield; $(S\times CBMY)_I$ and $(A\times CBMY)_j$ are the interactions of centered baseline milk yield with sulfite and acetate, respectively; C_m is the random effect of cow; and ε_{ijklm} is

the residual error. Least-squares means were separated using Tukey's adjustment when appropriate. Statistical significance was declared at $P \leq 0.05$, and tendencies were discussed at $0.05 < P \leq 0.10$.

RESULTS

Dry matter intake, Body weight and Body Condition Score

Cows receiving 0% sulfite in the diet had the highest DMI (29.1 kg/d), which was higher than cows supplemented with 0.45% sulfite (27.9 kg/d), and 0.9% sulfite (27.8 kg/d) (Table 4.2). Acetate supplementation did not affect DMI. Average DMI was similar between cows receiving no acetate (28.5 kg/d) and those receiving 1% acetate (27.9 kg/d) (Table 4.2). In addition, baseline milk yield had a significant effect on DMI (*Table 4.8*).

Dry matter intake showed a sulfite × week interaction, so treatment effects were interpreted within week (*Table 4.3*). In weeks 1 and 2, DMI did not differ significantly among sulfite levels ($P > 0.05$), although cows fed 0.45% sulfite tended to have numerically lower intakes than those fed 0 or 0.9% sulfite (27.1 vs. 28.0-28.4 kg/day). In week 3, DMI was greatest for cows receiving 0% sulfite (30.9 kg/day), intermediate for those receiving 0.5% sulfite (29.4 kg/day), and lowest for cows receiving 1% sulfite (27.4 kg/day), with 0 and 1% sulfite differing significantly and 0.5% sulfite not clearly different from either ($P < 0.05$).

The main effect of acetate level on DMI was not significant ($P > 0.05$), whereas the main effect of week was significant. Because the acetate × week interaction was also significant, DMI responses were interpreted within week. However, within each week DMI did not differ statistically between 0 and 1% acetate (*Table 4.4*).

Body weight (BW) was affected by sulfite inclusion in the cattle diet. Cows supplemented with 0.45% sulfite had the greatest body weight (690 kg), which differed from cows receiving no sulfite (674 kg), while cows receiving 0.9 % sulfite (676 kg) were intermediate (*Table 4.2*). In contrast, change in body weight (ΔBW) was not affected by sulfite supplementation, however, numerically, the cows in 0.9% sulfite included diet (16 kg) had greater change in body weight than 0 % (9 kg) and 0.45% sulfite (10kg) (*Table 4.2*).

In period 1 (653.72 kg) cows had the lowest body weight while body weight increased significantly in periods 2 (682.29 kg), 3 (691.19 kg), and 4 (693.61 kg) ($P < 0.0001$), with no differences among the latter three periods. Change in body weight (ΔBW) was also affected by period (*Table 4.5*). The body weight gain was higher during period 2 (30.97 kg), whereas weight change in periods 1 (1.67 kg) and 4 (4.05 kg) was lower. Weight change during period 3 (9.89 kg) was intermediate and did not differ from the other periods. Body condition score (BCS) was also not influenced by sulfite level, acetate and period.

Milk yield and efficiency

Milk yield was influenced by sulfite supplementation but was not affected by acetate supplementation (*Table 4.2*). Increasing sulfite level significantly decreased milk production. Cows fed diets without sulfite produced more milk (39.2 kg/d) compared with cows receiving 0.45% (37.9 kg/d) and 0.9% sulfite (36.4 kg/d) (*Table 4.2*).

Milk yield showed a significant week × sulfite interaction, so treatment effects were evaluated within week (*Table 4.8*). In week 1, milk yield did not differ significantly among sulfite levels, as LSMeans for 0, 0.45, and 0.9% sulfite were 39.2, 38.2, and 37.5

kg/day, respectively. In weeks 2 and 3, cows fed 0% sulfite produced more milk than those fed 0.45, and 0.9% sulfite (week 2: 39.5 vs. 37.9 and 37.1 kg/day; week 3: 39.1 vs. 37.9 and 36.8 kg/day) (*Table 4.3*). However, milk yield did not differ between the 0.45, and 0.9% sulfite treatments.

Period tended to affect milk yield, with cows producing the most milk in period 1 and progressively less in later periods (*Table 4.5*). ECM kg/day was highest in periods 1, 2, 3, and substantially lower in period 4 (44.60, 44.07, 42.67, and 38.79 kg/day for periods 1-4, respectively).

Feed efficiency (kg milk/kg DMI) was not affected by sulfite level, acetate supplementation, or experimental period. Feed efficiency remained same across the sulfite treatment that was 1.4 and 0.7 for acetate treatment. Across experimental periods, feed efficiency ranged from 1.3 to 1.5 kg milk/kg DMI without significant differences (*Table 4.2*). In addition, baseline milk yield had a significant effect on milk yield and efficiency (Table 4.8).

Milk composition and fatty acid profile

Sodium sulfite supplementation at 0, 0.45, and 0.9% DM did not alter milk composition ($P > 0.05$). Milk fat was 4.43%, 4.43%, and 4.26%; true protein was 3.14%, 3.18%, and 3.20%; lactose was 4.82%, 4.85%, and 4.88%; and milk urea nitrogen was 14.4, 14.7, and 14.5 mg/dL for the 0, 0.5, and 1.0% DM sulfite, respectively (*Table 4.6*).

Similarly, fatty acid fractions, including de novo fatty acids, mixed fatty acids, and preformed fatty acids, as well as total saturated, monounsaturated, and polyunsaturated fatty acids, were not influenced by sulfite supplementation. Individual fatty acids such as palmitic acid, stearic acid, and oleic acid, also showed no significant

differences among sulfite levels. Supplementation with acetate (1% of DM) did not affect milk composition or fatty acid concentrations compared with the control diet. Milk fat averaged 4.32% in control and 4.42% in 1% acetate, while true protein averaged 3.17% for both treatments. Lactose, urea, and fatty acid fractions also remained unchanged. Likewise, fatty acid groups (de novo, mixed, and preformed FA) and individual fatty acids (palmitic, stearic, and oleic acid) were not influenced by acetate supplementation.

Period effect

Tendency on milk yield, dry matter intake were observed significant period effects were observed for most milk composition and fatty acid parameters, while lactose remained unaffected. Milk fat percentage was significantly lower in Period 4 (4.03%) compared to Periods 2 and 3 (4.52% and 4.52%, respectively), while Period 1 (4.41%) showed intermediate values (*Table 4.7*). True protein concentration was highest in Period 1 (3.26%) and lowest in Period 2 (3.13%), with Periods 3 and 4 showing intermediate values of 3.11% and 3.19%, respectively. Lactose concentration remained stable across all periods, ranging from 4.82% to 4.88%. Milk urea nitrogen (MUN) decreased significantly in Period 4 (13.2 mg/dL) compared to Periods 1, 2, and 3 (14.8, 15.3, and 14.7 mg/dL, respectively; (*Table 4.7*).

Substantial period effects were observed in milk fatty acid composition. De novo fatty acids, which are synthesized in the mammary gland, were significantly lower in Period 4 (0.96%) compared to Periods 1, 2, and 3 (1.16%, 1.16%, and 1.15%, respectively) Mixed fatty acids, derived from both mammary synthesis and blood lipids, showed the highest concentration in Period 3 (1.41%) and the lowest in Period 4 (1.27%), with Periods 1 and 2 showing intermediate values of 1.35% and 1.37%, respectively.

Preformed fatty acids, which originate from circulating lipids, were highest in Period 3 (1.47%) and lowest in Periods 1 and 4 (1.28% for both), while Period 2 showed an intermediate value of 1.38%

Total unsaturated fatty acids were significantly lower in Period 4 (0.99%) compared to Periods 2 and 3 (1.11% and 1.17%, respectively), with Period 1 showing an intermediate value of 1.07%. Similarly, saturated fatty acids were lowest in Period 4 (2.54%) compared to Periods 2 and 3 (2.87% for both), while Period 1 had an intermediate concentration of 2.79%. Monounsaturated fatty acids showed a progressive increase from Period 1 to Period 3 (0.91%, 0.96%, and 1.02%, respectively), followed by a significant decrease in Period 4 (0.86%). Polyunsaturated fatty acids were highest in Period 3 (0.17%) and lowest in Period 4 (0.15%), with Periods 1 and 2 showing intermediate values.

Individual fatty acids also exhibited significant period effects. Palmitic acid (C16:0) concentration was numerically highest in Period 2 (1.33%) and lowest in Period 4 (1.21%), though the differences were less pronounced than other fatty acids. Stearic acid (C18:0) showed a progressive increase from Period 1 to Period 3 (0.39%, 0.42%, and 0.44%, respectively), followed by a significant decrease in Period 4 (0.35%). Similarly, oleic acid (C18:1), the predominant monounsaturated fatty acid in milk fat, increased from Period 1 to Period 3 (0.77%, 0.83%, and 0.88%, respectively) before decreasing significantly in Period 4 (0.73%) (*Table 4.7*).

DISCUSSION:

DM intake:

Dry matter intake was reduced with inclusion of sodium sulfite, which is consistent with previous beef-cattle feeding trial where intake was depressed. In that study, the authors attributed the lower intake to feed refusal caused by palatability issues, resulting in inconsistent intake patterns (van Nevel et al., 1973). However, in another dairy cow study where molasses was included with sulfite, no palatability problems were reported and dry matter intake remained similar between treatments (control 16.73 kg/day; sulfite 16.80 kg/day) (Alhassan et al., 1969). Intake decreased 4-6% therefore palatability could be one of the issues for reduced intake in sulfite included diet.

The decrease in dry matter intake in our experiment might be related to altered rumen fermentation during methane inhibition, with a shift toward increased propionate production. Because propionate is a glucogenic precursor, greater propionate supply can increase hepatic glucose availability and energy status, which may promote satiety and thus reduce voluntary feed intake (Allen et al., 2009). Another possible mechanism is the accumulation of H_2 in the rumen when methanogenesis is inhibited. Elevated dissolved H_2 inhibits the re-oxidation of NADH to NAD^+ via H_2 production under anaerobic conditions, making key NADH-oxidizing, H_2-producing reactions thermodynamically less favorable and kinetically slower (Kohn et al., 2000; Ungerfeld, 2020; Van Lingen et al., 2016). As a consequence, microbial metabolism becomes constrained and fiber degradation declines, which can further contribute to reduced dry matter intake and gut fill (Allen et al., 2009). Decreased intake may also be attributed to higher salt content on the Na_2SO_3 diets, as ruminants decrease intake on high salt diets.

The reduction in dry matter intake during the final week suggests that rumen microbes may have initially tolerated the highest level of sulfite supplementation.

However, prolonged exposure may have inhibited sensitive microbial populations, while allowing more resistant populations to persist under this stress condition (Khobragade et al., 2024). Consequently, extended sulfite exposure in the rumen may have impaired fiber-degrading bacteria, altered ruminal redox balance through increased H_2 accumulation, or enhanced sulfide production via assimilatory pathways (Daniels et al., 1986; Van'nevel et al., 1970). These changes may have collectively contributed to the depression in dry matter intake observed at the higher inclusion rate.

A minimal acetate effect on DMI was observed, with intake reduced by 2.5% when 1% acetate was included in the diet. However, the effect of acetate on intake has been inconsistent across studies. Some studies reported that ruminal infusion of acetate at rates of 18 mol/day and 10 mol/day reduced dry matter intake by 11% and by 10.2% to 14.3%, respectively (GMaxin et al., 2011; Urrutia et al., 2025). In contrast, dietary inclusion of acetate increased DMI by 2.7 kg/day in lactating cattle, with no change in milk yield (Urrutia et al., 2019).

The reason for reduced DMI with acetate inclusion is not clear; however, several assumptions have been proposed. One explanation is that continuous acetate supplementation may provide sufficient energy to extrahepatic cells, thereby reducing the need for propionate oxidation in the liver. This shift may promote short-term satiety signals and eventually reduce feed intake (Allen et al., 2009). Another explanation involves the relationship among acetate, butyrate, and insulin. Acetate increases the concentration of β-hydroxybutyrate (Storry & Rook, 1965) because it provides more acetyl-CoA for de novo fatty acid synthesis in mammary and peripheral tissue which may replace butyrate or other ketogenic substrates from oxidation, allowing more acetyl-CoA

to be directed toward ketogenesis and BHBA production (Allen et al., 2009). Consequently, butyrate stimulates insulin secretion in ruminants, which may further contribute to reduced DMI (Husveth et al., 1996).

Although the inclusion of acetate had no effect on DMI within week, the amount of DM intake for the 1% acetate group rose in week 3 relative to weeks 1 and 2, simultaneously similar trend was observed for the control group. These trends probably suggest an adaptive response by the cow and rumen to the additive in a time-dependent manner instead of a direct stimulating effect of acetate. Additionally, DMI and rumen microbial populations only adjusted when placed on a new diet for at least 2 to 3 weeks. Baseline milk yield was used as a covariate in order to control for possible variation in production ability among cows and showed significant impact on DMI. The above finding suggests that higher milk producers tended to ingest a larger amount of dry matter due to higher nutritional needs. Nevertheless, there was no significant interaction between baseline milk yield and either of the additives, both sulfite and acetate produced the same intake effects irrespective of initial milk production levels.

Body weight and BCS

Methane production represents a loss of metabolic energy from feed, because 2 to 12% of gross energy intake can be lost as enteric methane in dairy cows (Niu et al., 2018). When methane inhibitors inhibit methanogenesis, part of this energy loss can be diverted towards metabolic pathways in the animal (Morgavi et al., 2023). In the present study, sodium sulfite inclusion in a corn-based TMR resulted in the greatest body weight compared with the control diet. This response may reflect a combination of reduced energy losses as methane and altered rumen fermentation patterns. Methane-mitigation

strategies often increase ruminal propionate production, and propionate serves as a major gluconeogenic precursor in the ruminant liver (Wang et al., 2023). Increased propionate availability may enhance hepatic glucose synthesis, supporting tissue accretion and body weight gain (Orzuna-Orzuna & Lara-Bueno, 2023). Pedrini et al. (2024) reported that growing cattle supplemented with 3-NOP showed lower muscle pyruvate concentrations, a 6% increase in average daily gain, and improved carcass weight, suggesting that reduced methane losses may redirect carbon toward anabolic processes that favor body mass gain.

Another reason for greater body weight with the sulfite-supplemented rations could relate to water retention because the diet with Na_2SO_3 had more salt. Sodium sulfite is a salt, and each additional gram of salt in the diet can increase water intake by about 0.11 lb (Erickson, 2021). Because cows fed the sulfite diets received more salt than controls, the increased live BW could have reflected greater water intake and gut fill rather than true tissue accretion. However, body condition score which reflects body fat reserves rather than short-term changes in water balance was also numerically higher in the 0.5 and 1% sulfite groups than in the control. The parallel increase in BCS therefore suggests that BW gain in sulfite-fed cows represented an effect of the improved energy supply, and not solely a transient effect of salt-driven water retention. Additionally, the numerically higher change in bodyweight for sulfite group than control also indicated that the addition of sulfite helped to partition energy towards the body weight gain in the dairy cattle.

Acetate is the main oxidative fuel and a primary precursor for de novo fatty acid synthesis in ruminants, especially in adipose tissue and mammary gland. Previous studies

suggested that ruminal acetate does not directly determine body weight gain, whereas improved gain is more consistently associated with greater total VFA production, a lower acetate:propionate ratio, and higher-concentrate diets (Chen et al., 2021; Urrutia et al., 2025). In our study, cows were in mid-lactation, and body weight did not differ significantly among treatments, suggesting that acetate supplementation primarily supported milk fat synthesis with limited impact on overall body reserves. Nevertheless, body weight gain was numerically greater in cows fed the acetate-supplemented diet, which may reflect a small increase in energy retention. Because acetate is a major end product of rumen fermentation, additional dietary acetate altered VFA metabolism and increased the supply of glucogenic precursors through shifts in ruminal propionate production (Cheng et al., 2022). Scott (2022) also reported that inclusion of sodium acetate increased propionate concentration in vitro. Thus, increased propionate production may have enhanced the hepatic flux of gluconeogenic precursors, allowing surplus energy to be stored as tissue. Additionally, the numerically higher BCS in animals receiving acetate supplementation may further indicate that acetate supplementation partitioned more energy toward body tissue gain. However, measurements of total and individual VFA are needed to provide stronger evidence for shifts in ruminal fermentation and propionate production.

Milk production and Milk composition

Study hypothesized that inclusion of sulfite would increase milk yield by shifting ruminal fermentation toward propionate, in contrast cows fed sulfite produced less milk than controls. Because milk yield in lactating cows is closely related to energy and protein intake, the observed depression in DMI is expected to have contributed directly to

the lower milk yield in this treatment group. This response differs from previous work in which dietary sulfite did not alter DMI and consequently had no effect on milk production (Alhassan et al., 1969), suggesting that the negative impact of sulfite on milk yield in the present study is primarily mediated through reduced feed intake. In addition, the lack of any effect on milk production efficiency further supports the conclusion that reduced DM intake was the main driver of the milk production response. As week progressed the milk yield decreased in the sodium sulfite supplementation. The reduction in the milk yield could be due to decreased dry matter intake after first week.

The baseline milk yield was found to have a significant effect on milk yield, which means that those cows having higher baseline yields maintained higher yields during the entire experiment. There was no significant interaction between base-line milk yield and sulfite treatment in this experiment, which implies that sulfite had no influence on high and low milk yielding cows. On the other hand, the significant interaction between baseline milk yield and acetate suggests that acetate affected milk yield in different ways depending upon the baseline milk production level in those cows.

Although cows fed sulfite had lower DMI and produced less milk than controls, ECM yield was similar among treatments, indicating that milk energy density and the efficiency of energy use for lactation were largely maintained. Because cows in this study were in an energy-surplus stage of lactation, additional metabolizable energy could be partitioned both to body tissue accretion and to milk production. These results suggest that sulfite-induced changes in rumen fermentation and energy partitioning decreased milk volume but increased milk fat and/or protein concentration enough to preserve total milk energy output.

We found no effect of acetate on milk yield or ECM. This is consistent with other reports in which milk yield was unchanged when 2.7 kg/d of acetate were supplemented despite increased DMI (Urrutia et al., 2019). In contrast, studies using ruminal acetate infusions at 10 to 18 mol/d reported reduced milk yield, which coincided with decreased DMI (Maxin et al., 2011; Urrutia et al., 2025). Collectively, these findings suggest that milk yield is closely proportional to DMI; therefore, the absence of any change in DMI in acetate-fed cows relative to controls likely explains the lack of effect on milk production in the present study.

Milk components

Milk fat percentage was unchanged with the sulfite inclusion in animal diet was consistent with previous reports where fat percentage remained similar in sulfite group (3.05%) vs control groups (3.05%). Authors explained that, even though sulfite significantly improved propionate with reduction in the A:P ratio the magnitude of change was not enough to change the pathways for milk fat synthesis. However, several studies have found that decreased A:P contributed to the reduction of fat percentage due to lower availability of ruminal acetate for de novo fatty acid synthesis. True protein, lactose, and milk urea nitrogen concentrations were similar among cows fed 0, 0.45, or 0.9% sulfite on a DM basis. Likewise, acetate supplementation at 1.0% of dietary DM did not alter milk composition compared with the control diet, as fat, true protein, lactose, and urea concentrations remained unchanged.

Sulfite supplementation did not affect milk composition or milk fatty acid profile. Milk fat percentage was unchanged with the sulfite inclusion in animal diet was consistent with previous reports where fat percentage remained same in sulfite group

(3.05%) vs control groups (3.05%). Additionally, true protein, lactose, and urea concentrations were similar among cows fed 0, 0.45, and 0.9% sulfite of dietary DM, and proportions of de novo, mixed, and preformed fatty acids, as well as saturated, monounsaturated, polyunsaturated, palmitic, stearic, and oleic acids, were unchanged. Likewise, acetate supplementation at 1.0% of dietary DM did not alter milk composition or fatty acid profile compared with the control diet. These results indicate that changes in rumen fermentation induced by sulfite or acetate were too small to modify the supply of fatty acid precursors or the mammary enzymes regulating milk fat synthesis.

Period effect

Body weight increased after Period 1, consistent with previous reports indicating that cows regain body weight following early lactation as net energy balance becomes positive and dry matter intake increases (NASEM, 2021, Ellis et al., 2006). During the postpartum period, cow body weight can decline by approximately 10%, as high-producing cows rely extensively on mobilization of body fat reserves to meet the energy demands of milk synthesis (Peiter et al., 2023). In the present study, cows at the beginning of Period 1 were in the late early phase of mid-lactation, whereas Periods 2, 3, and 4 represented late mid-lactation through the initial phase of late lactation.

Body weight and BW change in the cows showed the expected pattern for cows transitioning through mid-lactation. Despite the highest feed intake cows had lowest body weight in period 1, it suggested that cows were still recovering from the negative energy balance from early lactation where with higher intake energy is partitioned into milk synthesis rather than body weight gain (NASEM, 2021, Ellis et al., 2006). Because, during the postpartum period, cow body weight can decline by approximately 10%, as

high-producing cows rely extensively on mobilization of body fat reserves to meet the energy demands of milk synthesis (Peiter et al., 2023).

In period 2, when cows were predominantly in mid-lactation, the tendency on improvement in the milk yield:DMI ratio suggests that animals were utilizing feed energy more efficiently for milk production and body weight gain, even though they consume less feed than in period 1. As days in milk increased across periods, DMI declined as expected and was accompanied by reduced body weight gain, indicating that cows were approaching early late-lactation in period 4, when the rate of tissue accretion normally slows to avoid over-conditioning while milk yield remains relatively stable or slightly declining. At this stage, cows have reduced energy requirements and are able to maintain a positive energy balance as milk yield declines (Ellis et al., 2006). During the prolonged lactation period, concentrations of insulin-like growth factor I, leptin, and glucose increase, whereas concentrations of growth hormone and non-esterified fatty acids decrease (Marett et al., 2011). These shifts in blood metabolites indicate that cows transition into an anabolic stage where intake gets affected.

The higher milk true protein percentage in period 1 may be partly explained by higher DMI during that period compared with the other periods. Milk protein synthesis depends on both the energy supply (primarily metabolizable energy) and the metabolizable protein (including microbial protein) available to the mammary gland (Kalscheur et al., 1999). With higher intake, cows generally have greater supplies of energy and metabolizable protein, which can support higher milk protein concentration; conversely, reduced intake in later periods may have contributed to the slightly lower protein percentages observed.

A period effect was observed for milk fat percentage. The lowest fat percentage in Period 4 is contrary to what is typically observed with physiological changes in dairy cattle, as milk fat concentration is generally higher during early and late lactation. Previous reports suggest that hot and humid climatic conditions can reduce milk fat percentage, partly due to decreased dry matter intake specially during summer vs winter or spring (Bernabucci et al., 2015; Looper, 2012). Because period 4 occurred from the third week of May to the first week of June, elevated ambient temperatures may have contributed to the reduction in milk fat percentage observed during this period.

De novo fatty acid synthesis was lowest in period 4, which may have contributed to the observed milk fat depression that led to reduced mixed FA%, palmitic acid% and saturated FA%. Reductions in de novo fatty acid synthesis usually occur due to changes in dietary composition, particularly diets high in starch or unsaturated fat, which promote the upregulation of alternative ruminal biohydrogenation pathways. These pathways produce intermediate fatty acids, such as trans-10 and cis-12 conjugated linoleic acid, which are known to play a critical role in diet-induced milk fat depression in dairy cows (Baumgard et al., 2002). Since cows received the same diet throughout the experiment, diet-induced milk fat depression is unlikely to explain the reduction in milk fat during Period 4. However, previous studies have demonstrated that even short-term heat stress (4 days) had significantly reduced de novo fatty acid synthesis (Z. Liu et al., 2017). Additionally, cow mobilize the adipose tissue during early days of lactation therefore, performed long-chain FA (C18:0, C18:1), mixed FA and MUFA increased early days in milk (Bielak et al., 2016), consistent with it the current study showed performed FA,

mixed FA (C16:0) and MUFA were higher during period 1-3 and decreased as days in milk progressed during period 4.

Summary

The study evaluated the effects of supplementing sodium sulfite (0, 0.45, and 0.9% of DM) and acetate (0 and 1% of DM) in the TMR diet of mid-lactating dairy cattle. The results showed that milk yield decreased in the sulfite-supplemented diets, whereas acetate had no effect on milk yield. Interestingly, body weight increased significantly in the sulfite-treated groups, particularly at 0.5% DM, despite reduced dry matter intake at both sulfite levels compared with the control. Acetate did not affect any performance parameters, including milk yield and milk fat synthesis. Contrary to our hypothesis, sulfite appeared to alter feed energy partitioning in a way that favored body weight gain. Therefore, future research should investigate different sulfite dose levels and their potential to promote body mass gain in beef cattle under long-term feeding conditions.

Table 4.1: Ingredient and nutrient composition of the lactating cow diet

Variable	Amount	DM or Concentration
Ingredient composition		
Corn silage trench 3 12-3-24	32.60 kg	9.30 kg DM
Triticale silage 12-30-24	4.29 kg	2.27 kg DM
Alfalfa haylage cut 1 12-30-24	1.51 kg	1.13 kg DM
Total forage	**38.41 kg**	**12.70 kg DM**
Corn, fine ground	6.30 kg	5.67 kg DM
ML04-12-23 UMD LACT RUM FLY	4.53 kg	4.15 kg DM
Soybean meal	2.54 kg	2.27 kg DM
Cottonseed, whole	1.59 kg	1.45 kg DM
Megalac	0.15 kg	0.15 kg DM
Total concentrate	**15.10 kg**	**13.68 kg DM**
Total ration	**53.51 kg**	**26.38 kg DM**
Nutrient composition		
Dry matter	13.00 kg	49.30%
Crude protein	4.50 kg	17.08%
RDP, % of CP	63.54	63.54
RUP, % of CP	37.09	37.09
Digestible Met	77.51 g	2.94 g/kg
Digestible Lys	197.30 g	7.48 g/kg
NDF	7.45 kg	28.24%
Forage NDF	4.93 kg	18.70%
Digestible NDF	3.55 kg	13.45%
Adjusted total starch	7.29 kg	27.66%
Rumen-digestible starch	4.88 kg	18.50%
Rumen-soluble sugar	1.06 kg	4.03%
Fat	1.14 kg	4.33%
Ash	1.90 kg	7.19%
Calcium	196.49 g	0.75%
Phosphorus	91.89 g	0.35%
Magnesium	85.44 g	0.32%
Potassium	332.79 g	1.26%
Sulfur	58.95 g	0.22%
Sodium	122.10 g	0.46%
Chloride	130.13 g	0.49%
DCAD	6491.73 meq	24.61 meq/100 g
Manganese	1668.97 mg	63.27 mg/kg

Zinc	2297.13 mg	87.08 mg/kg
Copper	544.92 mg	20.66 mg/kg
Iron	4308.61 mg	163.33 mg/kg
Selenium	9.92 mg	0.38 mg/kg
Vitamin A	157.94 KIU	5.99 IU/g
Vitamin E	1747.88 IU	66.26 IU/kg

Abbreviations: DM = dry matter; RDP = rumen degradable protein; RUP = rumen undegradable protein; CP = crude protein; Met = methionine; Lys = lysine; NDF = neutral detergent fiber; DCAD = dietary cation-anion difference.

Table 4.2: Effects of sodium sulfite and sodium acetate on body weight, body condition score, feed intake (as-fed basis), dry matter intake, milk yield, and feed efficiency (milk yield/DMI) in lactating dairy cows, adjusted for baseline milk yield and days in milk

	Sulfite Level (%DM)					**Acetate Level (%DM)**			
Parameters	**0**	**0.45**	**0.9**	**SEM**	***P*-value**	**0**	**1**	**SEM**	***P*-value**
BW (kg)	674[b]	690[a]	676[ab]	12.4	0.018	678	683	11.7	0.39
ΔBW (kg)	9.1	9.9	16	4.70	0.67	12.6	10.7	4.30	0.78
BCS	2.55	3.11	2.66	0.360	0.41	2.6	2.94	0.31	0.46
Feed intake (as fed) (kg/d)	58.5[a]	55.9[b]	55.8[ab]	1.36	0.0026	57.3	56.2	1.202	0.133
DMI (kg/d)	29.1[a]	27.9[b]	27.8[ab]	0.69	0.0045	28.5	28.0	0.60	0.12
Milk yield (kg/d)	39.3[a]	38.0[b]	37.1[b]	0.68	0.001	38.2	38.1	0.595	0.89
ECM/day (kg)	42.9	42.9	41.7	1.01	0.507	42.6	42.4	0.91	0.87
Milk/DMI (kg/kg)	1.44	1.41	1.40	0.049	0.6039	1.41	1.43	0.04	0.53

a-b 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).

Table 4.3: Effects of dietary sulfite level x week on milk yield and dry matter intake of dairy cows.

		Milk yield kg/day		DMI Kg/day	
Week	**Level of sulfite (%)**	**LSMean**	**SEM**	**LSMean**	**SEM**
1	0	39.2^{ab}	0.63	28.4^{bc}	0.63
1	0.45	38.2^{b}	0.62	27.1^{c}	0.62
1	0.9	37.5^{b}	0.64	27.9^{bc}	0.63
2	0	39.5^{a}	0.66	28.0^{bc}	0.66
2	0.45	37.9^{b}	0.65	27.1^{c}	0.66
2	0.9	37.1^{b}	0.67	28.1^{bc}	0.68
3	0	39.1^{a}	0.95	30.9^{a}	0.97
3	0.45	37.9^{b}	0.91	29.4^{ab}	0.95
3	0.9	36.8^{b}	0.9	27.4^{bc}	0.96

a-c Least squares mean within each column with different superscripts differ significantly (**P** < 0.05). **SEM** = standard error of the mean.

Table 4.4: Effects of dietary sodium acetate level x week on milk yield and dry matter intake of dairy cows.

		Milk yield **(kg/day)**		DMI (**kg/day)**	
Week	**Level of acetate (%)**	**LSMean**	**SEM**	**LSMean**	**SEM**
1	0	38.4	0.64	28.1^{bc}	0.65
1	1	38	0.63	27.5^{bc}	0.64
2	0	38.1	0.64	27.9^{bc}	0.65
2	1	38.2	0.64	27.5^{c}	0.66
3	0	38.3	0.63	29.6^{a}	0.65
3	1	37.8	0.64	28.8^{ab}	0.66

a-c Least squares means within each column with different superscripts differ significantly ($P < 0.05$). **SEM** = standard error of the mean.

Table 4.5: Effect of experimental period on body weight, BCS, feed intake (As fed kg), DMI, Milk yield (kg/day), and Milk/DMI (kg/kg) in lactating dairy cattle.

	Period					
Parameters	**1**	**2**	**3**	**4**	**SEM**	***P*-value**
BW (kg)	654[b]	682[a]	691[a]	694[a]	680	0.001
ΔBW (kg)	1.7[b]	31.0[a]	9.9[ab]	4.1[b]	5.7	0.003
BCS	3.02	2.41	2.79	2.87	0.4	0.72
Feed intake (kg/d)	59.8	56.7	55.8	54.6	1.53	0.07
DMI (kg/d)	29.5	27.9	27.7	27.9	0.77	0.05
Milk yield (kg/d)	39.5	38	37.8	37.1	0.75	0.09
ECM/day (kg)	44.6[a]	44.0[a]	42.7[a]	38.8[b]	1.13	0.0006
Milk/DMI (kg/kg)	1.33	1.45	1.44	1.45	0.05	0.12

a-b 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).

Table 4.6: Effect of sodium sulfite and sodium acetate supplementation on milk composition and fatty acid profile in lactating dairy cows.

	Sulfite Level (%DM)					Acetate Level (%DM)			
Parameters	**0**	**0.45**	**0.9**	**SEM**	***P*-value**	**0**	**1**	**SEM**	***P*-value**
Milk composition									
Fat (%)	4.43	4.43	4.26	0.71	0.57	4.32	4.42	0.66	0.32
True protein (%)	3.14	3.18	3.2	1.01	0.25	3.17	3.17	0.91	0.96
Lactose (%)	4.82	4.85	4.88	0.03	0.48	4.84	4.86	0.03	0.59
Urea (mg/dL)	14.4	14.7	14.5	0.11	0.62	14.5	14.6	0.1	0.79
Fatty acid composition (%)									
De novo FA	1.1	1.12	1.1	0.04	0.81	1.09	1.12	0.04	0.32
Mixed FA	1.39	1.36	1.31	0.04	0.26	1.34	1.37	0.03	0.37
Preformed FA	1.39	1.37	1.3	0.34	0.23	1.34	1.37	0.29	0.33
Unsaturated FA	1.11	1.1	1.05	0.03	0.48	1.08	1.09	0.03	0.59
Saturated FA	2.79	2.81	2.71	0.04	0.69	2.73	2.81	0.03	0.24
Monounsaturated FA	0.96	0.95	0.91	0.04	0.43	0.93	0.95	0.03	0.51
Polyunsaturated FA	0.16	0.16	0.16	0.03	0.86	0.16	0.16	0.02	0.16
Individual fatty acids (%)									
Palmitic acid (C16:0)	1.29	1.3	1.25	0.08	0.65	1.26	1.3	0.07	0.22
Stearic acid (C18:0)	0.41	0.4	0.39	0.03	0.44	0.39	0.4	0.02	0.42
Oleic acid (C18:1)	0.82	0.81	0.78	0.01	0.58	0.8	0.81	0.01	0.5

Table 4.7: Effect of period on milk composition, fatty acid composition, and individual fatty acid profile in lactating cow.

	Period					
Parameters	1	2	3	4	**SEM**	***P*-value**
Milk composition						
Fat (%)	4.41^{ab}	4.52^{a}	4.52^{a}	4.03^{b}	0.73	0.003
True protein (%)	3.26^{a}	3.13^{b}	3.11^{ab}	3.19^{ab}	1.14	0.003
Lactose (%)	4.82	4.88	4.84	4.86	0.03	0.68
Urea (mg/dL)	14.8^{a}	15.3^{a}	14.7^{a}	13.2^{b}	0.12	0.001
Fatty acid composition (%)						
De novo FA	1.16^{a}	1.16^{a}	1.15^{a}	0.96^{b}	0.05	0.001
Mixed FA	1.35^{ab}	1.37^{ab}	1.41^{a}	1.27^{b}	0.04	0.013
Preformed FA	1.28^{b}	1.38^{ab}	1.47^{a}	1.28^{b}	0.37	0.001
Unsaturated FA	1.07^{ab}	1.11^{a}	1.17^{a}	0.99^{b}	0.04	0.001
Saturated FA	2.79^{ab}	2.87^{a}	2.87^{a}	2.54^{b}	0.04	0.002
Monounsaturated FA	0.91^{bc}	0.96^{ab}	1.02^{a}	0.86^{c}	0.04	0.001
Polyunsaturated FA	0.17^{ab}	0.16^{ab}	0.17^{a}	0.15^{b}	0.03	0.002
Individual fatty acids (%)						
Palmitic acid (C16:0)	1.27	1.33	1.31	1.21	0.09	0.015
Stearic acid (C18:0)	0.39^{bc}	0.42^{ab}	0.44^{a}	0.35^{c}	0.03	0.001
Oleic acid (C18:1)	0.77^{bc}	0.83^{ab}	0.88^{a}	0.73^{c}	0.01	0.001

$^{a\text{-}b}$Means in the same row with different superscripts differ ($P < 0.05$).

Table 4.8: Fixed effects tests for Milk yield kg/day, Dry matter intake and feed efficiency.

Milk yield kg/day

Source	DF	F Ratio	Prob > F
Week	2	0.461139	0.63
Level of Sulfite*Week	4	0.61777	0.65
Level of Acetate*Week	2	1.007916	0.37
Centered Baseline Milk (kg)	1	47.60211	0.0001
Centered Days in milk	1	0.246235	0.62
Level of Sulfite* Centered Baseline Milk (kg)	2	1.414062	0.25
Level of Acetate* Centered Baseline Milk (kg)	1	0.79157	0.38

Dry matter intake kg (DMI)

Source	DF	F Ratio	Prob > F
Week	2	11.59898	0.0001
Level of Sulfite*Week	4	5.626961	0.0002
Level of Acetate*Week	2	0.349441	0.71
Centered Baseline Milk (kg)	1	7.040266	0.01
Centered Days in milk	1	0.004235	0.95
Level of Sulfite* Centered Baseline Milk (kg)	2	2.0809	0.13
Level of Acetate* Centered Baseline Milk (kg)	1	4.80082	0.03

Milk yield/DMI kg

Source	DF	F Ratio	Prob > F
Week	2	1.69156	0.18
Level of Sulfite*Week	4	1.746699	0.141
Level of Acetate*Week	2	0.406425	0.67
Centered Baseline Milk (kg)	1	3.9062	0.06
Centered Days in milk	1	0.749733	0.39
Level of Sulfite* Centered Baseline Milk (kg)	2	1.629139	0.20
Level of Acetate* Centered Baseline Milk (kg)	1	0.155915	0.69

Chapter 5: Effect of sodium sulfite or sodium acetate on ruminal gas and VFA parameters

ABSTRACT

Sodium sulfite is a reducing agent that can act as an alternative electron acceptor in the rumen and has been reported to decrease methane production and alter volatile fatty acid (VFA) profiles in cattle. However, the efficacy of the sodium sulfite in combination with additional feed additives such as acetate has not been reported. The objective of this study was to evaluate the effects of dietary sodium sulfite, provided in combination with sodium acetate or sodium bicarbonate, on ruminal gas composition, fermentation end products, pH, and redox potential (Eh) in lactating dairy cows. The study hypothesized that inclusion of sodium sulfite in a TMR ration would decrease methane and increase propionate. The four cannulated cows from the previous chapter were assigned to an unreplicated 4 × 4 Latin square with four dietary treatments: (A) bicarbonate alone, (B) bicarbonate plus sodium sulfite, (C) acetate alone, and (D) acetate plus sodium sulfite, with treatments rotated across four experimental periods in a design balanced for carryover effects. Rumen headspace gas and rumen fluid from the cannulated cows were sampled before feeding on days 0, 3, 7, 14, 17, and 20 of each period, and on day 17 at 0, 4, and 8 h post feeding to characterize diurnal patterns. Gas samples were collected via a modified cannula port and analyzed for hydrogen (H_2), methane (CH_4), and carbon dioxide (CO_2) using gas chromatography, whereas rumen fluid was analyzed for VFA by gas chromatography, and for pH and Eh using electrodes. The air had the effect on the CH_4, CO_2, CH_4/CO_2 fraction with no effect of air x treatment. Dietary treatment did not affect mean rumen H_2, CH_4, and CH_4/CO_2 ratio, while CO_2 fraction was increased in sodium bicarb (0.38) diet than treatments sodium bicarb + sulfite (0.29), sodium acetate (0.28) and sodium acetate + sulfite (0.31). The concentrations of acetate, propionate,

butyrate, total VFA, or the acetate:propionate ratio were unaffected by the treatments and there was no evidence for treatment effects on diurnal patterns of gas or VFA variables. In contrast, gas composition and fermentation parameters varied with experimental period and sampling time: CH_4, CO_2, and CH_4/CO_2 tended to be greater in later periods, VFA concentrations were lowest in period 2. VFA, pH and Eh showed significant diurnal effects, with the lowest pH and least negative Eh at 8 h after feeding. Under the conditions of this experiment, combining sodium sulfite with acetate or bicarbonate did not produce the hypothesized changes in rumen fermentation or gas composition in high-producing lactating cows.

Keywords: fermentation, gas, sulfite, VFA

INTRODUCTION

Sodium sulfite is a white, powdery, water-soluble reducing agent that becomes oxidized to sodium sulfate when released into water (Clough, 2014).. When added to ruminant diets, sodium sulfite has been found to alter the redox balance of the rumen, which may ultimately influence microbial diversity, particularly methanogens (Van'nevel et al., 1970). This finding has opened new avenues for its potential use as an alternative electron acceptor in the rumen to compete with hydrogenotrophs, especially methane producers. However, only a limited number of studies have investigated the use of sulfite as a feed additive in cattle diets to modulate rumen fermentation parameters, either individually or in combination with other additives.

A previous in vitro study conducted in our laboratory demonstrated a prominent effect of sodium sulfite in reducing methane production when included at 0.4 mM concentration with orchard hay. However, the rumen inoculum used in that study was obtained from a nonlactating Holstein cow. Therefore, it would be interesting to investigate the effect of sodium sulfite in lactating cows, where rumen fermentation patterns and energy demands differ significantly (Sofyan et al., 2017). An in vivo study on dairy cattle reported that supplementation of sulfite in the diet increased the molar proportion of propionate (from 18% to 23.7%) and butyrate, while acetate concentration decreased (from 65% to 55%) (Krabill et al., 1969). The reduction in acetate production negatively affected milk quality by decreasing milk fat synthesis (Mu et al., 2021). Because sodium sulfite alters the proportions of volatile fatty acids (VFAs), the inclusion of additives such as sodium acetate and sodium bicarbonate in combination with sodium

sulfite may serve as a strategy to modulate rumen fermentation toward a more desirable profile.

Acetate serves as the primary substrate for de novo lipogenesis in the mammary gland (Lin et al., 2022), whereas propionate and butyrate are major gluconeogenic and ketogenic sources of energy for the host animal (Rémond et al., 1995; Young, 1977). Acetate supplementation showed in the increment of de novo lipogenesis in the mammary glands which increased the milk fat synthesis without affecting total volume of milk production (Urrutia & Harvatine, 2017). Similarly, sodium bicarbonate has been shown to increase the molar proportion of acetate in goats and heifer while maintaining the overall integrity of the ruminal ecosystem by stabilizing rumen pH (Hadjipanayiotou, 1982; González et al., 2008). Therefore, the inclusion of acetate and bicarbonate together with sodium sulfite is expected to improve fermentation and animal performance parameters.

To date, no in vivo or in vitro studies have been conducted to investigate the effect of dietary inclusion of sodium sulfite as a methane inhibitor in combination with acetate or bicarbonate in lactating dairy cattle. Therefore, this study aimed to evaluate the effects of combining these feed additives on ruminal fermentation characteristics such as volatile fatty acids (VFAs), gas production, pH, and redox potential (Eh). It was hypothesized that sodium sulfite would increase the proportion of propionate while decreasing dry matter intake and methane production, whereas acetate and bicarbonate supplementation would help optimize the acetate-to-propionate ratio.

MATERIALS AND METHODS

Animal, housing and feeding

The experimental procedures were approved by the University of Maryland Institutional Animal Care and Use Committee (IACUC#- R-MAY-25-18) ahead of the starting date of experiment. A total of 32 lactating multiparous Holstein cows, including four cannulated animals, were housed individually in a bedded-pack barn with Calan feeding gates at the Central Maryland Research & Education Center (CMREC), University of Maryland, College Park. This chapter focusses on results from the four fistulated cows, and results of the feeding trial with all 32 lactating cows were presented in the preceding chapter. The cows ranged from 85 to 291 DIM at the start of the first treatment.

Animals were assigned to four dietary treatments in an unreplicated 4 × 4 Latin square design using the four cannulated cows. Treatment sequences were systematically rotated across experimental periods to ensure balanced treatment representation over time. The four treatments included: (A) bicarbonate alone, (B) bicarbonate combined with sodium sulfite, (C) sodium acetate alone, and (D) sodium acetate combined with sodium sulfite. Sodium bicarbonate 1% of DM, sodium acetate 0.9% of DM and sodium sulfite 0.45% of DM were added in corn silage based TMR. The four treatments were balanced for DCAD.

Throughout the experimental periods, all animals were fed a basal diet formulated as a total mixed ration (TMR; see Table 5.2). To minimize potential carryover effects between periods, treatment assignments were rotated such that each cow received

different dietary treatments in successive periods, following a balanced sequence across periods.

Rumen Sampling

Rumen samples, including enteric gas and rumen fluid for VFA, pH, and Eh analyses, were collected on multiple days and at various time points within a day. Sampling for gas and rumen fluid (VFA, pH, and Eh) was conducted on days 0, 3, 7, 14, 17, and 20 of each period. To evaluate diurnal effects of the treatments on ruminal parameters, samples on day 17 were collected at 0, 4, and 8 h relative to feeding. As described above, each treatment group included one cannulated cow; therefore, rumen samples were collected from the four cannulated cows.

Gas sampling

Each cannula was equipped with a hose barb (Everbilt ¼-inch barb x ¼-inch MIP brass adapter fitting) and secured on both sides using a steel washer (Everbilt ¼-inch stainless steel flat washer) along with a rubber gasket. A short piece of tubing (<2 inches) was connected to the brass barb using a hose clamp, and a two-way Luer-lock stopcock valve was attached to the open end of the tubing to allow for gas collection using a syringe. An extra piece of screw cap was placed on the open end of Luer-lock stopcock valve to prevent accidental leakage.

On the day of sample collection, cow was restrained in the chute area and rumen gas samples were collected first by opening the valve in the fistula cover. A 50 mL of syringe was connected with open end of Luer-lock stopcock valve and then flushed the syringe thrice at least before withdrawal of 50 mL of final gas sample. The syringe was also connected with Luer-lock stopcock valve. The rumen gas was later analyzed in Gas

Chromatography 6890.

VFA sampling

Once the rumen cannula plug was removed after taking gas sample, about 1.5 mL of rumen fluid were collected in 2 mL of Eppendorf tubes from each cow. Tubes with rumen fluid were placed immediately into ice box and later kept in -20°C freezer for further analysis in GC.

pH and Eh

pH was measured immediately after collecting rumen fluid samples using a pH meter fitted with a pH electrode. Another meter equipped with an oxidation-reduction potential (ORP) probe (Mettler Toledo ORP electrode InLab Redox Ag) was used to measure the redox potential of the rumen fluid. For this measurement, a minimum of 30 mL of rumen fluid was transferred into 50 mL centrifuge tubes, and the respective probes were inserted into the collected samples. pH readings were recorded within a minute, while Eh readings took at least 10 minutes to obtain accurate measurements. pH and Eh were mostly taken at the same time when VFAs were collected.

Data analysis

Rumen fermentation data, including volatile fatty acids (VFA), pH, and oxidation-reduction potential (Eh), were analyzed using a mixed-effects model. The model included treatment, period, day, and their interactions (treatment × period and treatment × day) as fixed effects. Cow was included as a random effect to account for repeated observations within animals. Day was treated as a repeated measure with cow × period as the subject, and a compound symmetry covariance structure was assumed. The gas response variables, including H_2, CH_4, and CO_2, were analyzed using a mixed

model. Treatment, period, day (or sampling time), treatment × day (or treatment × time), air, and treatment × air were included as fixed effects. Cow was included as a random effect. Repeated measurements over day (or sampling time) were accounted for using cow × period as the subject. Air and treatment × air were included only in the gas model, and sampling point represented day (day 0, 3, 7, 14, 17, and 20) or time (0, 4, and 8 hours) depending on the analysis.

$Y_{ijklmn} = \mu + T_i + P_j + S_k + (T\times S)_{ik} + A_m + (T\times A)_{im} + C_l + \varepsilon_{ijklmn}$

Y_{ijk} is the production response (gas, vfa, pH, and Eh)

μ is the overall mean

T_i *is* the fixed effect of the *i*th treatment

P_j *is* the fixed effect of the *j*th period

D_l is the fixed effect of the i_{th} day

$(T\times P)_{ij}$ is the treatment × period interaction

$(T\times D)_{il}$ is the treatment x day interaction

S_K= fixed effect of sampling point (day or time, depending on the analysis),

$(T\times S)_{ik}$ is fixed effect of treatment × sampling point interaction,

A_m is fixed effect of treatment × air interaction,

C_l is random effect of cow

ε_{ijklmn} is the residual error.

Least-squares means were separated using Tukey's adjustment when appropriate. Statistical significance was declared at $P \leq 0.05$, and trends were discussed at $0.05 < P \leq 0.10$.

RESULTS

Gas and Volatile fatty acid

The air had no effect on H_2 fraction while CH_4, CO_2 and CH_4/CO_2 were affected by the air presence. No interactions between treatment and headspace air (Trt × Air) were detected for any gas variable. Additionally, CO_2 fraction in bicarb was highest (0.38) than bicarb + sulfite (0.29), acetate (0.28) and acetate + sulfite (0.31) (*Table 5.3*). In contrast, gas composition tended to vary with experimental period. H_2 fraction showed no period effect whereas CH_4, CO_2 and CH_4/CO_2 fraction showed period effect in which fractions were increased for each after period 1 (

Table 5.4).

There were no differences among treatments for acetate, propionate, butyrate, A:P ratio, or total VFA concentration (Table 5.5) and no diurnal effect on VFA parameters were observed (*Table 5.6*). However, a significant period effect was observed for all parameters. Acetate and propionate concentrations were lowest in Period 2 (27 mM and 15 mM, respectively) compared with Periods 1, 3, and 4 (*Table 5.6*). Similarly, butyrate concentration was lowest in Period 2 (6 mM) compared with Period 1 (11 mM) and Period 3 (12 mM), while it increased slightly in Period 4 (9 mM).

pH and Eh

There was no overall effect of treatment on pH or Eh. Redox potential (Eh) tended to decrease in Period 2 (−230 mV) compared with Periods 1, 3, and 4 (*Table 5.6*). A significant hour effect was observed for pH and Eh, with the lowest pH and less negative reducing potential at 8 h compared with the other sampling hours (Figure 5.1).

The post feeding hours effect was observed in the individual VFA too, all three major VFA increased by hours (Figure 5.1 – Figure 5.2).

DISCUSSION

Gas and Volatile Fatty Acid

The air composition within headspace influenced the percentage of CH_4 and CO_2, although since the interaction between treatment and air was not significant, it is evident that these differences remained similar regardless of whether the air composition was present or not. Alhassan et al. (1969) reported that inclusion of sodium sulfite in the diet of lactating dairy cows increased the concentration of highly reduced end products at the expense of surplus H_2 due to its inhibitory effect on methanogenesis. However, inconsistent with previous results our current study found no effect of sulfite on rumen gas as well as VFA parameters except CO_2 fraction. The addition of $NaHCO_3$ increases the bicarbonate pool size and buffering capacity to offer more HCO_3^-. HCO_3^- that will react with the H^+ from VFA, leading to the release of more CO_2 without necessarily affecting fermentation (CO_2 (d) + $H_2O \rightleftharpoons H_2CO_3 \rightleftharpoons HCO_3^- + H^+$) (Laporte-Uribe, 2019). The previous study reported that methane production decreased by 19.2% while H_2 accumulation increased up to 2.8% with 0.8% sulfite included diet in wether sheep vs control. The unchanged headspace gas concentration reflected in the VFA concentration too. Because individual VFA concentration were unaffected even in the sulfite included in this study. In contrast to our in vitro trail and previous reports (Alhassan et al., 1969; van Nevel et al., 1973; Van'nevel et al., 1970) sodium sulfite inclusion did not affect VFA parameters, including acetate, propionate, butyrate and valerate. Moreover, no changes in gas parameters such as H_2 and CH_4 explain the unchanged VFA production

observed in this trial. Because the inhibitory effect of sulfite on methanogens was expected to shift H_2 utilizing pathways towards propionate production and shifting fermentation away from acetate.

In in vitro study, the fermentation of fiber was delayed in the presence of sulfite and was nearly fully recovered within 24 hours. Since we collected the sample before feeding in the early morning, the microbes might have recovered after adjusting a few hours of sulfite in the rumen environment and the effect might have been masked. Because in vitro incubations do not contain rumen wall or other host-related processes. There is no absorption of VFA, no interaction with epithelial cells, no rumen contractions, and the feed-to-ruminal fluid ratio and retention time are very different from those in vivo (K. Wang et al., 2024). Because of this, a compound such as sulfite can remain at a higher effective concentration in vitro and may show a clearer dose-response effect. In vivo, sulfite may still have short-term effects on rumen redox conditions or microbial activity, but these effects are likely moderated by passage rate, absorption, saliva buffering, and normal feeding behavior. In addition, because sodium sulfite is a salt, water intake might increase in the animal, which could further dilute sulfite in the rumen. As a result, treatment effects observed in vivo are often smaller at the time of sampling.

Also, we collected the samples on day 17 at different time intervals; treatments had no effect on any of the VFA concentration. Microorganisms in the rumen may have the ability to adapt and develop a mechanism to degrade sulfite and produce energy for their growth. Even though we collected the samples at different time intervals on that day, the lack of an effect of sulfite could be due to microbial adaptability to this condition

for long exposure to sulfite. Jespersen & Wagner (2023) reported that some methanogenic microbes can detoxify and reduce sulfite rapidly by using F420-dependent sulfite reductase (Fsr) to sulfide or sulfur for their own growth.

After cows consumed the TMR rich in starch and sugars, readily fermentable carbohydrates became available to amylolytic and saccharolytic bacteria, stimulating rapid fermentation and VFA production. While fiber-degrading bacteria (e.g., *Ruminococcus, Fibrobacter, Butyrivibrio spp.*) start degradation of structural carbohydrates, provide glucose and other hexoses for microbial glycolysis and subsequently produce fermentation end products such as acetate, propionate, and butyrate (Raffrenato et al., 2022). The ruminal VFA concentration at any given time point reflects the balance between VFA production and clearance from the rumen. During the early hours after feeding, animals continue to consume feed, increasing the supply of substrate available for microbial degradation and fermentation. As a result, VFA production may exceed absorption, leading to VFA accumulation in the rumen. Therefore, VFA concentration reached at peak after 4 hours.

The period effect has been observed in most of the VFA parameters. Interestingly, period 2 had the lowest concentration for acetate, propionate, and butyrate and total VFA. The reduced concentrations of individual VFAs during Period 2 may be partly attributed to variation in GC peak identification or calibration caused by the use of a different VFA standard during sample analysis. As a result, period effect could have been observed. After period 2, the production of these individual VFA improved and maintained VFA concentration till period 4. There was no treatment effect on total VFA, and A:P, however, total VFA had period effect similar as individual VFA.

pH and Eh

No change in ruminal pH among treatments suggests ruminal stability. Ruminal pH depends on diet composition, microbial activity, and fermentation end products such as volatile fatty acids (Wu et al., 2024; F. Zhang et al., 2022) . It has been reported that an increase in total VFA concentration decreases ruminal pH. According to the Henderson-Hasselbalch equation, when the concentration of the undissociated form of an acid (HA) increases relative to the dissociated form (A^-), the pH decreases. This occurs because the pKa of acetate is approximately 4.8. Since total VFA concentration did not differ among treatments in the present study, this explains the stable pH observed throughout the experiment. However, the lack of change in pH with sodium acetate supplementation compared with the control diet may be attributed to the chemical behavior of acetate. When sodium acetate dissolves in water, it dissociates completely and can generate OH^- ions, which tend to make the solution more basic (Neavyn et al., 2013). Only an hour effect was observed for ruminal pH. Typically, ruminal pH is higher before feeding than after feeding due to microbial adaptation and the onset of fermentation once the animal consumes feed. Immediately after feeding, fermentation results in rapid accumulation of volatile fatty acids, which subsequently decreases ruminal pH. However, pH stabilizes once hydrogen ion (H^+) production and buffering reach equilibrium (Mao & Wang, 2025). Since the lowest ruminal pH typically occurs approximately 10 -14 hours after feeding (Zebeli et al., 2008), pH stabilization in the present study may have occurred at or after 8 hours post-feeding.

The reducing potential of the rumen is indicated by redox potential (Eh, mV), and a typical ruminal fluid Eh ranges from -440 to -50 mV (Friedman et al., 2017). More

negative Eh reflects a strongly anaerobic environment. We hypothesized that inclusion of sodium sulfite would alter ruminal Eh, making it less negative than the control. However, sodium sulfite supplementation in the TMR diet had no effect on Eh across treatments. Hydrogen partial pressure and pH have inverse relationships with reducing potential (Rietmann et al., 2023a). Since neither H_2 concentration nor pH differed among treatments, this explains why Eh was not affected by treatment. In the present study, ruminal Eh was most negative at 4 h post feeding, indicating the greatest reducing conditions in the rumen fluid at this time. Higher rate of fermentation of dietary starch and sugars generates substantial amounts of reducing equivalents (e.g., NADH) and metabolic hydrogen, thereby driving the redox potential to more negative values (Rietmann et al., 2023b). As time after feeding progressed, fermentable substrate was depleted and hydrogenotrophic microbes such as methanogens removed H_2 from the system, reducing the pool of available reducing equivalents in the rumen fluid. Consequently, Eh became less negative by 8 h post feeding, reflecting a shift toward a less reducing (more oxidized) environment.

Summary

In our study, we observed that adding sodium sulfite to the cow's diet did not change the rumen fermentation characteristics, such as gas production (H_2 and CH_4) and volatile fatty acid profiles. Similarly, when we supplemented sodium acetate along with sodium sulfite, we did not see any improvements in milk production or other performance parameters. These findings were quite different from what previous researchers have reported. Earlier studies suggested that sodium sulfite could help reduce methane emissions and alter rumen fermentation patterns. However, our results did not support

these claims. There could be several reasons for this discrepancy. First, the dosage we used in our experiment might not have been high enough to produce noticeable effects. Second, it is possible that the rumen microorganisms metabolized the sulfite too quickly, preventing it from influencing the fermentation process.

Based on these observations, we believe that additional research is needed to determine the optimal inclusion rate of sodium sulfite and to examine its effects under different dietary conditions. Future studies should investigate various dosage levels and dietary composition to better understand whether sodium sulfite can effectively reduce methane production and enhance propionate formation in lactating dairy cattle.

Table 5.1: Allocation of 4 treatment in 4 groups of cannulated cows across four period in 1 X 1 Latin Square design.

Group	Period 1	Period 2	Period 3	Period 4
1	d	c	b	a
2	c	a	d	b
3	b	d	a	c
4	a	b	c	d

Prior to initiating Period 1, 14-days adjustment phase was implemented during which all experimental cows consumed the basal total mixed ration. Dietary formulations were developed in accordance with National Research Council (NRC, 2003) standards to satisfy or surpass the nutritional needs of lactating dairy cattle.

Table 5.2: Ingredient and nutrient composition of the lactating cow diet

Variable	Amount	DM or Concentration
Ingredient composition		
Corn silage trench 3 12-3-24	32.60 kg	9.30 kg DM
Triticale silage 12-30-24	4.29 kg	2.27 kg DM
Alfalfa haylage cut 1 12-30-24	1.51 kg	1.13 kg DM
Total forage	**38.41 kg**	**12.70 kg DM**
Corn, fine ground	6.30 kg	5.67 kg DM
ML04-12-23 UMD LACT RUM FLY	4.53 kg	4.15 kg DM
Soybean meal	2.54 kg	2.27 kg DM
Cottonseed, whole	1.59 kg	1.45 kg DM
Megalac	0.15 kg	0.15 kg DM
Total concentrate	**15.10 kg**	**13.68 kg DM**
Total ration	**53.51 kg**	**26.38 kg DM**
Nutrient composition		

Dry matter	13.00 kg	49.30%
Crude protein	4.50 kg	17.08%
RDP, % of CP	63.54	63.54
RUP, % of CP	37.09	37.09
Digestible Met	77.51 g	2.94 g/kg
Digestible Lys	197.30 g	7.48 g/kg
NDF	7.45 kg	28.24%
Forage NDF	4.93 kg	18.70%
Digestible NDF	3.55 kg	13.45%
Adjusted total starch	7.29 kg	27.66%
Rumen-digestible starch	4.88 kg	18.50%
Rumen-soluble sugar	1.06 kg	4.03%
Fat	1.14 kg	4.33%
Ash	1.90 kg	7.19%
Calcium	196.49 g	0.75%
Phosphorus	91.89 g	0.35%
Magnesium	85.44 g	0.32%
Potassium	332.79 g	1.26%
Sulfur	58.95 g	0.22%
Sodium	122.10 g	0.46%
Chloride	130.13 g	0.49%
DCAD	6491.73 meq	24.61 meq/100 g
Manganese	1668.97 mg	63.27 mg/kg
Zinc	2297.13 mg	87.08 mg/kg
Copper	544.92 mg	20.66 mg/kg
Iron	4308.61 mg	163.33 mg/kg
Selenium	9.92 mg	0.38 mg/kg
Vitamin A	157.94 KIU	5.99 IU/g
Vitamin E	1747.88 IU	66.26 IU/kg

Abbreviations: DM = dry matter; RDP = rumen degradable protein; RUP = rumen undegradable protein; CP = crude protein; Met = methionine; Lys = lysine; NDF = neutral detergent fiber; DCAD = dietary cation-anion difference.

Table 5.3: Effect of Bicarbonate, Bicarbonate + Sulfite, Acetate and Acetate + Sulfite on individual ruminal gas fraction and CH_4/CO_2 in dairy lactating cows.

	Treatment						P-value	
		Bicarb +		Acetate				
Parameters	**Bicarb**	**Sulfite**	**Acetate**	**Sulfite**	**SEM**	**P-value**	**Air**	**Trt×Air**
H_2 Fraction	0.002	0.002	0.003	0.002	0.0019	0.4528	0.2765	0.0006
CH_4 Fraction	0.19	0.17	0.21	0.19	0.010	0.282	0.0867	0.5464
CO_2 Fraction	0.78	0.78	0.78	0.78	0.020	0.1285	0.6068	0.2749
CH_4/CO_2	0.24	0.25	0.27	0.25	0.010	0.3103	0.0048	0.5617

Table 5.4: Effect of period on individual ruminal gas fraction and CH_4/CO_2 in dairy lactating cows.

	Period						P-value	
Parameter	**1**	**2**	**3**	**4**	**SEM**	**P-value**	**Day**	**Trt×Day**
H_2 Fraction	0.004	0.000	0.000	0.006	0.0008	0.1992	0.8975	0.9948
CH_4 Fraction	0.17	0.21	0.20	0.21	0.005	0.0961	0.2942	0.0641
CO_2 Fraction	0.72	0.79	0.81	0.79	0.018	0.0278	0.0125*	0.0074*
CH_4/CO_2	0.21	0.28	0.26	0.27	0.033	0.0654	0.5164	0.6891

[ab]Means within a row with different superscripts are significantly different ($P < 0.05$).

Table 5.5: Effect of Bicarb, Bicarb + Sulfite, Acetate and Acetate + Sulfite on pH, Eh, individual ruminal VFA, total VFA, and A:P in dairy lactating cows.

	Treatment					
Parameters	**Bicarb**	**Bicarb + Sulfite**	**Acetate**	**Acetate + Sulfite**	**SEM**	**P-value**
pH	6.3	6.5	6.4	6.5	0.34	0.34
Eh	-252	-273	-244	-276	1.8	0.20
Acetate (mM)	46	46	47	42	2.8	0.36
Propionate (mM)	27	27	28	25	3.0	0.74
Butyrate (mM)	9	10	9	9	0.8	0.71
A:P	1.8	1.8	1.8	1.8	0.1	0.68
Total VFA (mM)	89	80	84	77	6.4	0.57

[a-b] Means in the same row with different superscripts differ (P < 0.05).

Table 5.6: Effect period on individual ruminal pH, Eh, acetate, propionate, butyrate, A:P and total VFA in dairy lactating cows.

	Period						P-value	
Fraction	**1**	**2**	**3**	**4**	**SEM**	**P-value**	**Day**	**Trtxday**
pH	6.4	6.5	6.4	6.4	0.12	0.5888	0.0866	0.58
Eh	-269	-230	-279	-269	3.7	0.0683	0.0079	0.65
Acetate (mM)	55[a]	27[b]	53[a]	46[a]	2.8	0.0002	0.2792	0.48
Propionate (mM)	33[a]	15[b]	32[a]	26[a]	3.0	0.0018	0.1832	0.68
Butyrate (mM)	11[a]	6[b]	12[a]	9[a]	0.8	0.0007	0.3338	0.70
A:P	1.7	1.9	1.7	1.8	0.1	0.1414	0.2752	0.96
Total VFA (mM)	99[a]	48[b]	96[a]	81[a]	6.5	0.0004	0.2193	0.54

[a-b] Means in the same row with different superscripts differ (P < 0.05).

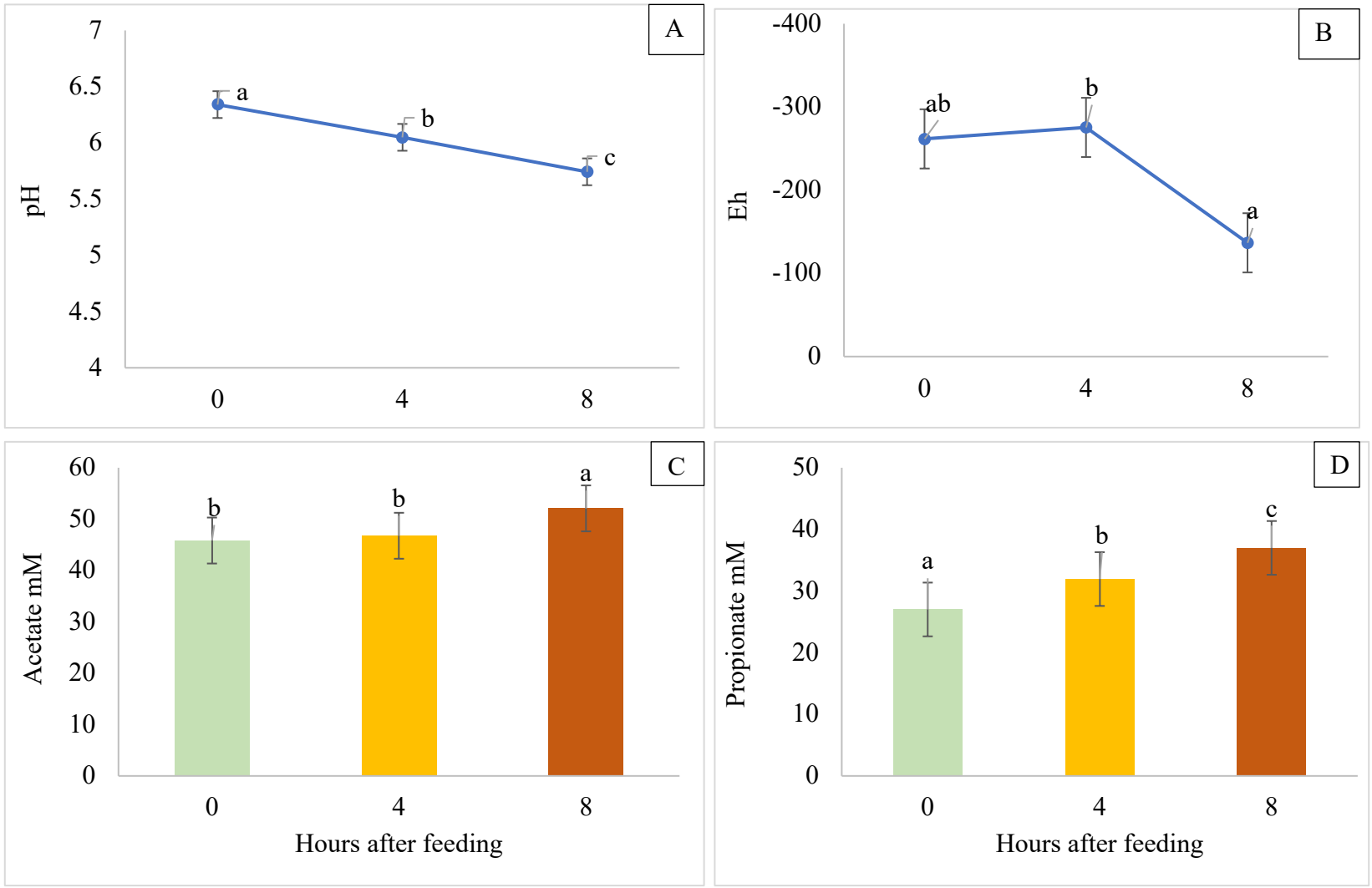


Figure 5.1: Effect of time on (A) pH, (B) Eh, and (C) acetate mM, and (D) propionate mM in dairy lactating cow, ($P<0.05$)

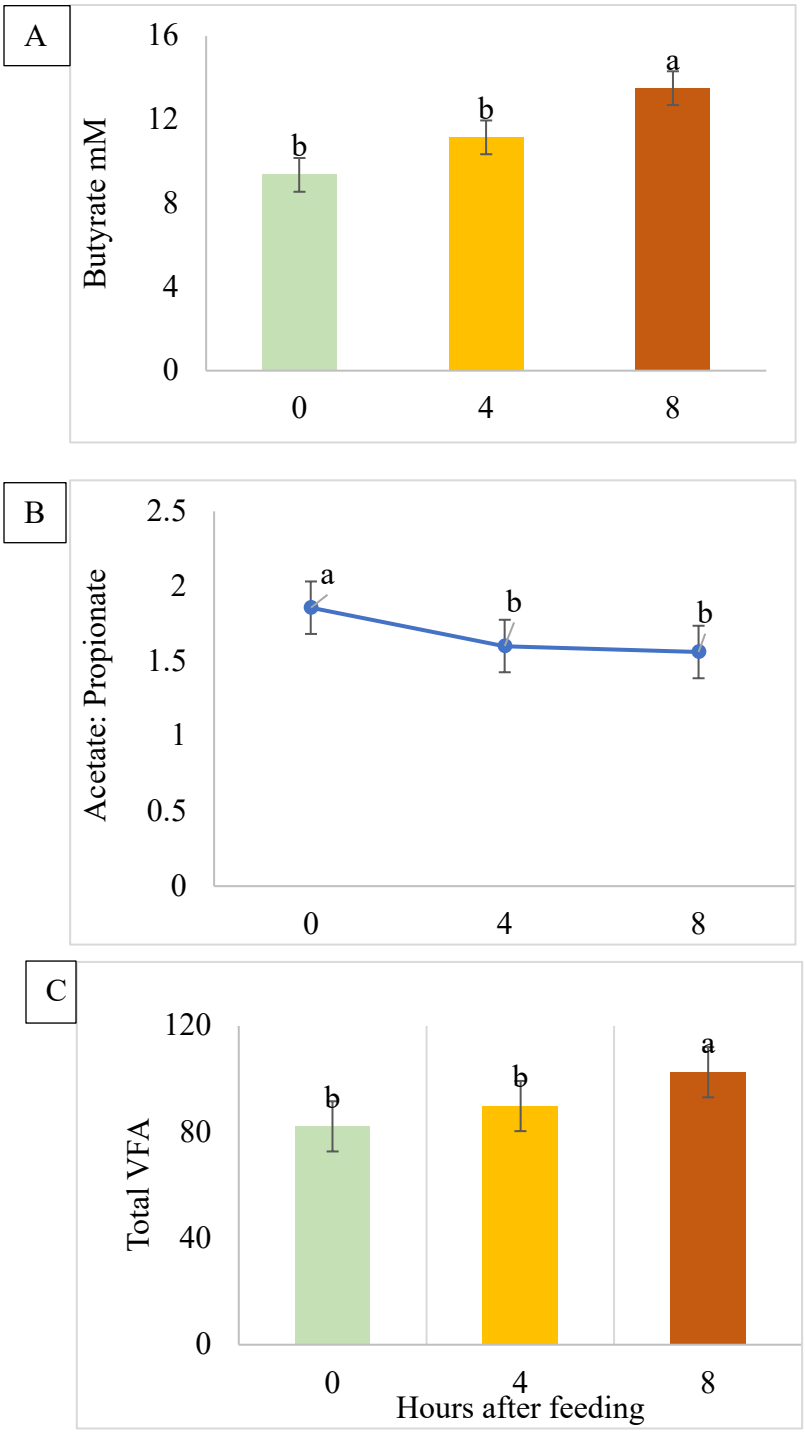


Figure 5.2: Effect of post feeding hours on (A) butyrate, (B) A:P, and (C) total VFA concentration in dairy lactating cattle, ($P<0.05$).

Chapter 6: Does adaptation to sodium sulfite in the rumen attenuate its effect on methane emissions.

ABSTRACT

Sodium sulfite has the potential to redirect metabolic hydrogen away from methanogenesis, but the extent and persistence of this effect may depend on the duration of exposure of rumen microbes to this compound. In the current experiment we investigated adaptability of the rumen microbes when exposed with for 2 weeks of dietary sodium sulfite supplementation. Rumen fluid was obtained from four rumen-cannulated Holstein cows enrolled in a 4 × 4 Latin square trial with dietary treatments of sodium bicarbonate, sodium bicarbonate + sodium sulfite, sodium acetate, and sodium acetate + sodium sulfite. Fifty milliliters of rumen fluid from each individual cow were added to 160-mL Erlenmeyer flasks containing one of the following treatments: blank, blank + sulfite, hay (0.5 g timothy), or hay + sulfite. The flasks were incubated in a shaking water bath at 39°C for 24 h. Fermentation gas was collected in Mylar balloons attached to each flask and sampled using a gas-tight plastic syringe at 4, 8, and 24 h. At each gas sampling time, 1.5 mL of rumen fluid was collected from each flask into 2-mL centrifuge tubes for VFA. Gas and VFA were analyzed using a 6890-gas chromatograph in the laboratory.

Total and cumulative gas production were not affected among donor dietary treatments. Net production of H_2 at 8 h was highest in acetate (0.024 mmol) compared with bicarbonate (0.017 mmol) whereas Acetate + sulfite (-0.014 mmol) and bicarbonate + sulfite (-0.004 mmol) showed negative values compared ($P = 0.0036$). In contrast, at 24 h, net production of H_2 in acetate + sulfite (-0.003 mmol) and bicarbonate + sulfite (-0.001 mmol) was greater compared with acetate (-0.022 mmol) and bicarbonate (-0.017 mmol; $P = 0.0034$).

The donor diet did not influence total VFA and acetate:propionate ratios; however, propionate at 8 h tended to be higher in acetate + sulfite (28 mM) compared with acetate (24 mM), bicarbonate + sulfite (19 mM), and bicarbonate (20 mM; $P = 0.081$). In contrast, the addition of sulfite in vitro significantly impacted gas dynamics. The sulfite reduced the amount of gas produced at 4 h (137 vs. 125 mL for the control), as well as the cumulative gas produced at 4 h and 8 h (125 vs. 137 mL and 143 vs. 157 mL, respectively; $P < 0.05$). Net H_2 produced at 4 h increased from 0.004 mmol in the control to 0.021 mmol with the sulfite addition ($P = 0.0001$). However, the net H_2 at 24 h was less negative with the sulfite addition compared with the control (-0.018 vs. -0.005 mmol; $P = 0.018$). Cumulative H_2 production was also greater with sulfite at 4 h (0.022 vs. 0.004 mmol; $P = 0.0001$) and 8 h (0.027 vs. 0.011 mmol; $P = 0.0174$). Net CH_4 production was less with sulfite than with the control at 4 h (0.011 vs. 0.059 mmol; $P < 0.005$) and 24 h (0.153 vs. 0.210 mmol; $P < 0.005$). The cumulative amount of CO_2 produced in the control was higher than in the sulfite treatment at 8 h (2.99 vs. 2.50 mmol), and there were tendencies towards sulfite effects on net CO_2 and cumulative CO_2 at 4 h ($P = 0.087$ and 0.086), respectively. There were tendencies towards decreased acetate in the sulfite treatment compared to the control at 4 and 8 h, and towards decreased acetate to propionate ratio in the sulfite treatment compared to the control over time ($P < 0.05$). Collectively, these results suggest that 2 weeks of in vitro sulfite exposure in the diet did not completely remove the antimethanogenic response to sulfite in vitro; rather, sulfite remained an alternative electron sink, increasing H_2 accumulation, decreasing CH_4 production, and affecting fermentation patterns, especially acetate, in a transient manner.

Keywords: fermentation, gas, sulfite, VFA

INTRODUCTION

Sulfur-containing compounds such as sulfate, sulfide, and sulfite have the ability to compete with hydrogenotrophic microorganisms for reducing equivalents in the rumen. During microbial reduction, sulfate is converted to hydrogen sulfide, a highly reduced end product in the rumen environment. Early studies demonstrated that sodium bisulfite can act as an alternative electron sink by counteracting with acetaldehyde, thereby suppressing the ethanol-forming pathway. As a consequence, acetaldehyde no longer accepts hydrogen, leading to increased glycerol formation as a terminal product (Gunsalus & Stanier, 1961). Because $SO^{2}_{3}{}^{-}$ can be reduced to S^{2-} or intermediates as it accepts the electron from highly reduced donor such hydrogen, formate, and organic acids. Collectively, these findings suggest that sodium sulfite possesses a unique capacity to regulate electron balance in the rumen, ultimately influencing gas production and volatile fatty acid (VFA) profiles in ruminants.

Short-term experiments including in vitro batch cultures (Alhassan et al., 1969) and rumen infusion studies (Van'nevel et al., 1970) showed that adding sulfite to rumen fluid decreased methane production but no effects on methane were observed in rumen fistulated cows fed sulfite for 21-d periods (Chapter 5). The inclusion of sodium sulfite in cattle diets did not affect dry matter intake, milk yield, or milk components, such as milk protein and fat percentage (Alhassan et al., 1969). However, these experiments were conducted over a short duration, and therefore the effects of prolonged dietary exposure to sodium sulfite remain unclear.

The rumen is a highly diverse and adaptable microbial ecosystem. The inclusion of, or changes in, dietary feed components can rapidly shift fermentation pathways during the initial phase, whereas adaptation to prolonged feeding of the same diet may reduce the magnitude of dietary effects on rumen fermentation parameters. Guo et al. (2022) reported that monensin supplementation initially reduced ruminal microbial diversity, but diversity recovered after 30 days, even though monensin supplementation continued. This study indicates that the rumen harbors both feed-additive-sensitive and feed-additive-resistant microbial populations, which can adapt over time and reduce the effectiveness of the additive.

The inclusion of feed additives such as sodium bicarbonate and sodium acetate, along with sodium sulfite, could influence microbial activity by partitioning hydrogen into different metabolic pathways. These buffering additives may alter ruminal pH, redox potential, and fermentation pathways. In addition, the microbes themselves may begin digesting these compounds and producing different end products upon long-term exposure. Therefore, testing microbial adaptability is important to evaluate the efficacy and longevity of these products for extended use. The response of sulfite on gas and VFA production in vitro may differ depending on the rumen fluid obtained from cows fed different combinations of diets.

The objective of this study was to validate the adaptability of rumen microorganisms to dietary supplementation with sodium sulfite. The study hypothesized that the external addition of sodium sulfite in vitro to rumen cultures obtained from rumen-fluid donor cows receiving sodium sulfite-based treatments would not result in

changes in gas or VFA production, whereas rumen cultures from cows not previously exposed to sulfite would show measurable responses.

MATERIALS AND METHOD

Animal, housing and feeding

The experimental procedures were approved by the University of Maryland Institutional Animal Care and Use Committee (IACUC#- R-MAY-25-18) ahead of the starting date of experiment. Four Holstein cows fitted with rumen cannulas were individually housed in a bedded-pack barn equipped with Calan gate feeding systems at the University of Maryland's Central Maryland Research & Education Center (CEMREC) in Ellicott City, Maryland. The four cows were part of a larger study with intact animals described in Chapter 4 and 5. Animals were assigned to four treatment groups in a 4 × 4 Latin square design, with treatments systematically rotated across experimental periods. The dietary treatments included: bicarbonate alone, bicarbonate + sodium sulfite, sodium acetate alone, and sodium acetate + sodium sulfite. Measurements from fistulated cows followed a single Latin square design. All experimental cows received a basal total mixed ration (TMR) throughout the study. The experimental animals progressed from early to late mid-lactation during the study period.

Rumen fluid collection

Rumen fluid on day 14 was collected from each cow in each period. The rumen fluid was collected in 50-mL tubes after milking and prior to feeding and immediately transferred into a blender jar under continuous CO_2 flushing. For the in vitro study, we established a laboratory at the CEMREC. After homogenization, the rumen fluid was

strained through four layers of cheesecloth with glass wool on top and transferred into an Erlenmeyer flask. The rumen fluid was mixed thoroughly with the help of a stir bar and a magnetic stir plate.

The 160-mL Erlenmeyer flasks containing 0.5 g of timothy were prepared one day in advance, sealed with rubber stoppers, and secured with wire. Each stopper contained two glass tubes: one connected to a Mylar balloon for collection of fermentation gases and the other used for rumen fluid sampling for VFA analysis.

Rumen fluid was added to randomly assigned flasks, followed by the addition of 1.6 mL of the respective treatment solution. The in vitro treatments included: blank, hay, sodium sulfite, and hay + sodium sulfite. These treatment combinations were applied to rumen fluid collected from cows receiving four dietary treatments: sodium bicarbonate, sodium bicarbonate + sodium sulfite, sodium acetate, and sodium acetate + sodium sulfite.

Gas and fluid sample collection

The in vitro bottles were incubated in a hot water shaker bath maintained at 39 °C in the university laboratory. Gas volume was measured at 4, 8, and 24 h using a 100-mL airtight Fortuna glass syringe, and gas samples were collected in 10-mL plastic syringes fitted with two-way stopcock valves. Fluid samples for VFA analysis were collected simultaneously with gas sampling. The VFA samples were immediately stored at −20 °C for subsequent gas chromatography (GC) analysis, whereas gas samples were analyzed immediately by GC to determine gas composition.

Gas equation

Total gas production, cumulative gas production, net individual gas production, change in individual gas production, cumulative, rate of individual gas formation were calculated based on the following equation:

$$V_t = V_{s,t} + V_h \ldots\ldots\{1\}$$

$$V_{cum,t} = V_h + \sum_{t=0}^{t} V_{s,t} \ldots\ldots\ldots\{2\}$$

$$X_t = \frac{(V_{s,t} \times C_t) + V_h (C_t - C_{t-1})}{22.4} \ldots\ldots\ldots\{3\}$$

$$X_{cum,t} = X_t + \frac{\sum_{t=0}^{t} (X_{t-1})}{22.4} \ldots\ldots\ldots\{4\}$$

Where,

V_t = volume of gas (mL) at time t

$V_{cum,t}$ = Cumulative gas production at time t (mL)

X_t = Net production of individual gas (CH_4, H_2 and CO_2) at time point (t) (mmol)

$X_{cum,t}$ = Cumulative individual gas production at time t (mmol)

$V_{s,t}$ = Volume of gas collected in syringe at time t, and

V_h = Volume of headspace

Vs_{t-1} = Volume (mL) of gas in syringes in previous time point

C_t = Concentration of individual gas at time t and 22.4 is the molar volume of an ideal gas at STP.

C_{t-1} = Concentration of individual gas in previous time point (t-1)

X_{t-1} = Production of individual gas (CH_4, H_2 and CO_2) at time point (t-1) (mmol)

Statistical analysis

The data obtained from the in vitro incubation test was analyzed using the Mixed Model procedure in JMP software (Student Edition, SAS Institute Inc., Cary, NC, USA). The variables used in the analysis were the gas and VFA obtained from the in vitro flasks. As the analysis was done for individual time points, the variable 'time' was used as a by variable in the JMP software, with a separate analysis done for each individual time point.

The cow effect was also included as a random effect to accommodate the repeated rumen fluid sampling from the cow. In addition, flask effect was specified as the repeated effect, and repeated measures were specified with a compound symmetry covariance structure, with cow-period specified as the subject. This covariance structure is used to accommodate the correlated data collected from the same experimental unit within periods. Model below was used with length of time in vitro incubation as a by variable. The statistical model used for each response variable was:

$$Y_{ijkl}=\mu+D_i+A_j+P_k+(D\times A)_{ij}+C_l+\varepsilon_{ijkl}$$

where:

Y_{ijkl} = dependent variable measured in vitro;

μ = overall mean;

D_i = fixed effect of the *i*th donor diet;

P_k = fixed effect of the *k*th period;

A_j = fixed effect of the *j*th added in vitro treatment;

$(D\times A)_{ij}$ = interaction between donor diet and added in vitro treatment;

C_l = random effect of cow;

ε_{ijkl} = residual error.

Least squares means were generated for fixed effects, and differences among means were considered significant at $P \leq 0.05$, whereas $0.05 < P \leq 0.10$ was considered a tendency.

RESULTS

Effects of donor diets

Total gas and cumulative gas production did not differ among donor diets. Net hydrogen production at 8 hours was highest in acetate (0.024 mmol), followed by bicarb (0.017 mmol), whereas acetate + sulfite (-0.014 mmol) and bicarb + sulfite (-0.004 mmol) diets yielded negative net values (Table 6.1). At 24 hours, net hydrogen production was greater for acetate + sulfite (-0.003 mmol) and bicarb + sulfite (-0.001 mmol) compared with acetate (-0.022 mmol) and bicarb (-0.017 mmol) (*Table 6.2*). Negative values indicate H_2 usage by that time point. The donor diet had no effect on the any of the VFA parameters including total VFA as well as A:P ratio (Table 6.7). There was tendency on propionate mM at time point 8 hours where propionate was higher in acetate + sulfite (28 mM) and followed by acetate (24 mM), bicarb+ sulfite (19 mM), and bicarb (20 mM) (

Effects of in vitro treatment

Sulfite addition in vitro reduced total gas at 4 hours (125 mL) compared with the control (137 mL). The cumulative gas was greater in the control (137 and 157 mL) than

in the sulfite-treated incubations (125 and 143 mL) at 4 and 8 hours, respectively ($P<0.05$), with a tendency for a similar difference at 24 hours (*Table 6.4*). Inclusion of sulfite in vitro increased net H_2 production at 4 hours (0.021 mmol) relative to the control (0.004 mmol) and hydrogen was subsequently consumed up to 24 hours in the sulfite treatment; consequently, net H_2 production at 24 hours was higher in the control (-0.005 mmol) than in the sulfite treatment (-0.018 mmol) (*Table 6.5*). However, cumulative H_2 production was greater with the sulfite-added diet at 4 hours (0.022 vs 0.004 mmol) and 8 hours (0.027 vs 0.011 mmol) compared with the control (Table 6.6).

Net methane production was lower with sulfite addition (0.011 and 0.153 mmol) than in the control (0.059 and 0.21 mmol) at 4 and 24 hours, respectively ($P<0.005$) (Table 6.5). Cumulative CH_4 production decreased from the beginning to the end of the fermentation period, and was consistently lower with sulfite than in the control at 4, 8, and 24 hours (0.012, 0.165, and 0.428 mmol vs 0.059, 0.264, and 0.562 mmol) (*Table 6.6*). At 8 hours, cumulative CO_2 production was higher in the control (2.99 mmol) than with sulfite addition (2.50 mmol). A tendency was observed for effects of sulfite on net CO_2 and cumulative CO_2 at 4 hours (Table 6.5 - 6.6).

The in vitro inclusion of sulfite had no effect on individual VFAs. A tendency was detected for acetate concentration at 4 and 8 hours, where sulfite inclusion reduced acetate compared with the control (*Table 6.8*). The acetate-to-propionate ratio was consistently lower with sulfite (2.2 vs 2.4) and remained at this ratio at 4, 8, and 24 hours (*Table 6.8*).

Donor treatment x In vitro sulfite

At 4 and 8 h, cumulative H_2 showed only tendencies for a donor diet × in vitro sulfite interaction, and no effect at 24 h (*Table 6.9*), indicating that early differences in H_2 were small and largely disappeared by the end of incubation. Within acetate-based donor diets, bottles receiving in vitro sulfite consistently produced numerically more H_2 at 4 and 8 h than those without in vitro sulfite, regardless of whether sulfite was included in the donor diet. A similar pattern was observed for bicarbonate-based donor diets, where the combination with in vitro sulfite was also associated with greater early H_2 production compared with the corresponding treatments without in vitro sulfite, again with differences diminishing by 24 h. In contrast, cumulative CH_4 at 4 h showed a significant donor diet × in vitro sulfite interaction, whereas interactions at 8 and 24 h were not detected, indicating that sulfite mainly altered methanogenesis during the early phase of incubation. Within acetate-containing donor diets, the greatest 4-h CH_4 production occurred with Acetate + Sulfite without in vitro sulfite, whereas inclusion of in vitro sulfite in either Acetate or Acetate + Sulfite reduced early CH_4. Within bicarbonate-based donor diets, in vitro sulfite inclusion likewise decreased 4-h CH_4 relative to the corresponding treatments without in vitro sulfite. At 8 h of incubation, bottles inoculated with rumen fluid from acetate or Acetate + Sulfite-based donor diets and incubated without in vitro sulfite produced greater cumulative CO_2 than their respective counterparts receiving in vitro sulfite, whereas no donor diet × in vitro sulfite interaction effects on cumulative CO_2 were detected at 4 or 24 h. No interaction effect were observed for VFA parameters (*Table 6.10*).

DISCUSSION

Gas parameters

The rumen fluid already exposed to sodium sulfite through the cattle diet had no effect on total gas or cumulative gas production. However, a previous report found that infusion of sodium sulfite (5 grams daily) directly into the rumen of wethers decreased total gas production from 30.3 mL to 12.4 mL (Van'nevel et al., 1970). In the current study, at 8 h, the net H_2 production, cumulative H_2, and the rate of H_2 formation were significantly different among the various donor diets. The net H_2 production was positive in the acetate and bicarb inoculum at 8 h. This indicates that in these systems, the interaction of in vitro-added sulfite and hydrogen-consuming microbes, such as methanogens, inhibited H_2 production initially, thereby maintaining the positive net H_2 production. In the Acetate + Sulfite and Bicarb + Sulfite inoculum, which were previously exposed to sulfite, the microbes may have been adapted to utilize the sulfite via sulfate-reducing microbes. Which may have reduced the sulfite to hydrogen sulfide and other sulfur-containing compounds that promote the growth of microbes (Jespersen & Wagner, 2023). However, with further exposure, the hydrogen sulfide produced in higher concentration may have inhibited the reduction of sulfite and H_2 consumption become inefficient in hydrogen utilizing pathways (Ungerfeld, 2020)

The sulfite added in vitro initially reduced the total gas. The presence of sulfite in vitro likely exerted an inhibitory effect on rumen fermentation, consistent with earlier work using sodium sulfite to manipulate ruminal fermentation patterns (Krabill et al., 1969a). Since sulfite directly inhibit major hydrogen utilizing pathways that methanogens the thermodynamic conditions for further H_2 production become less favorable, which

can slow fermentation and reduce total gas output. This observation agrees with reports that sulfite inclusion decreases total gas and methane production in vitro and alters the stoichiometry of fermentation end products (Krabill et al., 1969a).

There are three primary pathways for methane formation in the rumen: hydrogenotrophic, methylotrophic, and acetoclastic pathways (Waters et al., 2025). Previous studies have reported that the hydrogenotrophic pathway serves as the major sink for H_2 through the reduction of CO_2 to CH_4 (Khairunisa et al., 2023; Waters et al., 2025) . The exact mechanism by which sulfite inhibits methanogenesis is not yet fully understood. However, Sulfite (SO_3^{2-}) is a strong nucleophile and reactive sulfur intermediate it disrupts electron transfer reactions inside microbial cells and collapses their central energy metabolism, leading to inhibition of methane production (Jespersen & Wagner, 2023). The significant rise in hydrogen levels and decrease in methane at during the initial stage of fermentation suggests that sulfite directly inhibited hydrogenotrophic methanogens when added in vitro.

Consistent with previous findings van Nevel et al. (1973), net CH_4 production decreased and hydrogen production increased sharply with the inclusion of sodium sulfite in vitro at time point 4 hours in the current study. However, as fermentation progressed, the methane inhibition became less pronounced, and no difference occurred in net H_2 production at time point 24 hours. This suggests that although sodium sulfite initially competes for hydrogen or temporarily inhibits methanogen activity, the microbial population can adapt and resume normal function at later stages of fermentation.

Despite sulfite (SO_3^{2-}) being inhibitory or toxic to methanogens, it can also serve as a sulfur source for several species, including *Methanococcus thermolithotrophicus* and

Methanobacterium thermoautotrophicum, and can support growth (Daniels et al., 1986) . This is because these methanogens express high levels of F420-dependent sulfite reductase on sudden expose to sulfite, which metabolizes sulfite and protects against sulfite toxicity (Johnson & Mukhopadhyay, 2008). This process converts sulfite into a more reduced form of sulfur, that is sulfide, thereby facilitating microbial growth.

There was only a tendency for treatment effects on CO_2 production, and cumulative CO_2, in vitro. With sulfite, CO_2 production decreased compared with the no-sulfite treatment during the initial stage of incubation, whereas by 24 h CO_2 production was reversed and became even higher than the control. This pattern suggests that sulfite affected not only methanogens but also primary fermenters, transiently depressing fermentation activity, which subsequently recovered after 24 h.

In contrast to our hypothesis that inclusion of sodium sulfite together with different feed additives would lead to microbial adaptation and thus no further response to additional sulfite, however, the present in vitro gas measurements indicated that sulfite still influenced fermentation. Specifically, donor diet and in vitro sodium sulfite showed measurable effects on gas parameters, most notably on CH_4 production, which decreased when sulfite was added in vitro to both acetate- and bicarbonate-based donor diets. This pattern suggests that the rumen microbiota in these incubations had not fully adapted to sulfite, but rather that sulfite continued to interfere with normal methanogenic activity and slightly delayed fermentation. Consistent with the role of sulfite as alternative hydrogen sinks, these in vitro gas results supports that sulfite acted as a competing sink for metabolic hydrogen, transiently increasing H_2 accumulation, decreasing early CH_4,

and modestly altering CO_2 production without compromising overall fermentation extent by the end of incubation.

Volatile fatty acids

The donor diet showed no effect on acetate, butyrate, or total VFA; however, there was a tendency for an effect on propionate, with the acetate + sulfite donor diet having a greater propionate concentration than the others. This could have occurred due to a shift in H_2-utilizing pathways toward propionate production (Ungerfeld, 2020b) , especially at 8 h, when net H_2 gas production was negative, suggesting that H_2 was being captured by propionate-producing microbes. However, this effect was transient, and propionate concentration changed only slightly after this time point.

The in vitro inclusion of sulfite showed a tendency to reduce acetate concentration, which aligns with a previous report where the molar percentage of ruminal acetate decreased from 65% to 55% (Krabill et al., 1969a). Because H_2 accumulated initially as methanogenesis was reduced, fermentation became thermodynamically less favorable, which slowed overall fermentation and resulted in lower acetate production. Acetate-producing pathways release reducing equivalents (H_2 or reduced cofactors) that normally flow to methanogens; sulfite provides an alternative electron sink (sulfite → sulfide) (Hedderich et al., 1998) while simultaneously inhibiting methanogenesis, so conditions become less favorable for high acetate yield and for strictly acetogenic/fibrolytic microbes.

In contrast to a previous report (van Nevel et al., 1973), propionate concentration was unchanged in sulfite-treated in vitro incubations despite increased H_2 production. Because sulfite is a electron acceptor (Hedderich et al., 1998), it may promote

sulfite/sulfate-reducing bacteria that compete with propionate producers for metabolic hydrogen, resulting in no net change in propionate concentration. The lower acetate:propionate ratio in sulfite-amended in vitro incubations was therefore due to reduced acetate production. Since propionate concentration was not even numerically higher than in the control, the lower ratio reflected decreased acetate rather than increased propionate in the sulfite-supplemented diet.

Summary

In this study rumen fluid was tested from animals that had been exposed to sulfite or non-sulfite diets. The in vitro results indicated that rumen microbes were not adapted to sulfite even after 2 weeks of in vivo exposure: when sulfite was added in vitro, hydrogen accumulation increased and methane production was markedly reduced in a similar pattern irrespective of the donor diet. This inhibition was also evident in acetate-producing microbes, resulting in lower acetate production. Furthermore, rumen inoculum from animals fed different diets responded differently in terms of hydrogen production, particularly during the initial stage of fermentation. Overall, sulfite inclusion, whether in vivo or in vitro, affected rumen fermentation only transiently, and rumen microbes appeared to return to normal activity after a few hours of exposure.

Table 6.1: The effect of donor diet on total gas at 1 atm. measured at each time step (gas in excess of bottle headspace volume was discarded after measurement) and cumulative gas production at different time points 4, 8 and 24 hours in vitro.

Total gas mL

Donor Diet	4 HR	8 HR	24 HR	SEM
Bicarb	126	88	103	5.2
Bicarb + Sulfite	133	96	117	5.1
Acetate	123	92	113	5.2
Acetate + Sulfite	140	97	113	5.1
P- value	0.4917	0.279	0.4203	

Cumulative gas mL

Donor Diet	4 HR	8 HR	24 HR	SEM
Bicarb	126	138	165	10.7
Bicarb + Sulfite	133	154	196	10.5
Acetate	123	144	181	10.5
Acetate + Sulfite	140	163	201	10.4
P- value	0.4917	0.4151	0.457	

The total gas (mL) and cumulative gas (mL) were derived using equation 1 and 2.

[a-b] Means within a row not connected by the same letter are significantly different ($P < 0.05$).

Table 6.2: The effect of donor diet on net production between time steps of H_2 mmol, CH_4 mmol and CO_2 mmol production at different time points 4, 8 and 24 hours in vitro.

Net H_2 production mmol

Donor Diet	4 HR	8 HR	24 HR	SEM
Bicarb	0.011	0.017[ab]	-0.017[b]	0.0047
Bicarb + Sulfite	0.01	-0.004[bc]	-0.001[a]	0.0047
Acetate	0.01	0.024[a]	-0.023[b]	0.0046
Acetate + Sulfite	0.021	-0.014[c]	-0.003[a]	0.0043
P- value	0.5392	0.0036	0.0034	

Net CH_4 production mmol

Donor Diet	4 HR	8 HR	24 HR	SEM
Bicarb	0.02	0.126	0.227	0.0305
Bicarb + Sulfite	0.033	0.199	0.284	0.0285
Acetate	0.034	0.161	0.316	0.0292
Acetate + Sulfite	0.055	0.23	0.36	0.0283
P- value	0.3819	0.3826	0.2585	

Net CO_2 production mmol

Donor Diet	4 HR	8 HR	24 HR	SEM
Bicarb	2.252	0.218	1.284	0.2356
Bicarb + Sulfite	2.274	0.527	1.231	0.2325
Acetate	2.272	0.746	1.429	0.2392
Acetate + Sulfite	3.06	0.415	1.425	0.2305
P- value	0.3355	0.7164	0.6814	

The net individual gas production mmol were derived using equation 3

Values indicate net change in gas between timesteps. Negative values indicate consumption of gas type.

[a-b]Means within a row not connected by the same letter are significantly different ($P < 0.05$).

Table 6.3: The effect of donor diet on cumulative H_2 mmol, CH_4 mmol and CO_2 mmol production at different time points 4, 8 and 24 hours in vitro.

Cumulative H_2 mmol

Donor Diet	4 HR	8 HR	24 HR	SEM
Bicarb	0.011	0.029[ab]	0.009	0.0044
Bicarb + Sulfite	0.01	0.006[b]	0.004	0.0044
Acetate	0.01	0.034[a]	0.009	0.0042
Acetate + Sulfite	0.021	0.007[b]	0.004	0.004
P- value	0.53	0.01	0.33	

Cumulative CH_4 mmol

Donor Diet	4 HR	8 HR	24 HR	SEM
Bicarb	0.02	0.15	0.338	0.0481
Bicarb + Sulfite	0.033	0.232	0.5	0.0467
Acetate	0.034	0.193	0.499	0.0471
Acetate + Sulfite	0.055	0.285	0.644	0.0465
P- value	0.38	0.33	0.27	

Cumulative CO_2 mmol

Donor Diet	4 HR	8 HR	24 HR	SEM
Bicarb	2.252	2.424	3.533	0.3538
Bicarb + Sulfite	2.274	2.684	3.895	0.3432
Acetate	2.272	2.409	3.919	0.3529
Acetate + Sulfite	3.06	3.475	4.899	0.3417
P- value	0.33	0.27	0.26	

Cumulative individual gas production (mmol) was calculated from equation 5.

[a-b]Means within a row not connected by the same letter are significantly different ($P < 0.05$).

Table 6.4: The effect of added sulfite in vitro on total gas at 1 atm. measured at each time step (gas in excess of bottle headspace volume was discarded after measurement) and cumulative gas (mL) production at different time points 4, 8 and 24 hours in vitro.

Total gas mL

In vitro Sulfite	4 HR	8 HR	24 HR	SEM
NO	137[a]	95	110	2.1
YES	125[b]	92	112	2.2
P- value	0.0002	0.2992	0.4816	

Cumulative gas mL

In vitro Sulfite	4 HR	8 HR	24 HR	SEM
NO	137[a]	157[a]	192	4.2
YES	125[b]	143[b]	179	4.4
P- value	0.0002	0.006	0.07	

[a-b] Means within a column not connected by the same letter are significantly different ($P < 0.05$).

Table 6.5: The effect of added sulfite in vitro on net production between time steps of H_2 mmol, CH_4 mmol and CO_2 mmol production at different time points 4, 8 and 24 hours in vitro.

Net H_2 production mmol

In vitro Sulfite	4 HR	8 HR	24 HR	SEM
NO	0.004[b]	0.007	-0.005[a]	0.0038
YES	0.022[a]	0.005	-0.018[b]	0.0038
P- value	0.0001	0.70	0.02	

Net CH_4 production mmol

In vitro Sulfite	4 HR	8 HR	24 HR	SEM
NO	0.059[a]	0.205[b]	0.306	0.0147
YES	0.012[b]	0.153[b]	0.287	0.0159
P- value	0.0001	0.70	0.01	

Net CO_2 production mmol

In vitro Sulfite	4 HR	8 HR	24 HR	SEM
NO	2.664	0.389	1.16	0.113
YES	2.265	0.564	1.524	0.1197
P- value	0.09	0.37	0.11	

Values indicate net change in gas between timesteps. Negative values indicate consumption of gas type.

[a-b] Means within a column not connected by the same letter are significantly different ($P < 0.05$).

Table 6.6: The effect of added sulfite in vitro on cumulative H_2 mmol, CH_4 mmol and CO_2 mmol production at different time points 4, 8 and 24 hours in vitro.

	Cumulative H_2 mmol			
In vitro Sulfite	**4 HR**	**8 HR**	**24 HR**	**SEM**
NO	0.004[b]	0.011[b]	0.006	0.0033
YES	0.022[a]	0.027[a]	0.007	0.0033
P- value	0.0001	0.02	0.85	

	Cumulative CH_4 mmol			
In vitro Sulfite	**4 HR**	**8 HR**	**24 HR**	**SEM**
NO	0.059[a]	0.264[a]	0.562[a]	0.017
YES	0.012[b]	0.166[b]	0.428[b]	0.0184
P- value	0.0001	0.0002	0.004	

	Cumulative CO_2 mmol			
In vitro Sulfite	**4 HR**	**8 HR**	**24 HR**	**SEM**
NO	2.664	2.994[a]	4.144	0.139
YES	2.265	2.501[b]	3.979	0.1524
P- value	0.09	0.02	0.59	

Negative values indicate consumption of gas type.

[a-b] Means within a column not connected by the same letter are significantly different ($P < 0.05$).

Table 6.7: The effect of donor diet in vitro on acetate mM, propionate mM, butyrate mM, A:P, and total VFA mM concentration at different time points 4, 8 and 24 hours in vitro.

Acetate mM

Donor diet	4 HR	8 HR	24 HR	SEM
Bicarb	40	44	46	5.6
Bicarb + Sulfite	45	50	59	5.6
Acetate	49	56	66	5.6
Acetate + Sulfite	51	59	63	5.6
P-value	0.43	0.23	0.39	

Propionate mM

Donor diet	4 HR	8 HR	24 HR	SEM
Bicarb	19	20	22	2.5
Bicarb + Sulfite	19	21	26	2.5
Acetate	21	24	29	2.5
Acetate + Sulfite	25	28	29	2.5
P-value	0.17	0.08	0.32	

Butyrate mM

Donor diet	4 HR	8 HR	24 HR	SEM
Bicarb	7	8	9	1.3
Bicarb +Sulfite	9	10	12	1.3
Acetate	8	11	13	1.3
Acetate + Sulfite	11	13	14	1.3
P-value	0.23	0.18	0.37	

A:P

Donor diet	4 HR	8 HR	24 HR	SEM
Bicarb	2.4	2.33	2.243	0.13
Bicarb + Sulfite	2.45	2.42	2.387	0.13
Acetate	2.37	2.4	2.313	0.13
Acetate +Sulfite	2.07	2.14	2.119	0.13
P-value	0.2078	0.3086	0.2701	

Total VFA mM

Donor diet	4 HR	8 HR	24 HR	SEM
Bicarb	67	72	77	9.1
Bicarb + Sulfite	73	82	97	9.1
Acetate	78	91	107	9.1
Acetate + Sulfite	86	100	106	9.1
P-value	0.33	0.17	0.38	

Table 6.8: The effect of added sulfite in vitro on acetate mM, propionate mM, butyrate mM, A:P, and total VFA mM concentration at different time points 4, 8 and 24 hours in vitro.

		Acetate mM		
In vitro Sulfite	**4 HR**	**8 HR**	**24 HR**	**SEM**
NO	48	55	60	2.9
YES	44	50	57	2.9
P- value	0.08	0.06	0.24	

		Propionate mM		
In vitro Sulfite	**4 HR**	**8 HR**	**24 HR**	**SEM**
NO	21	24	26	1.9
YES	21	23	27	1.9
P- value	0.95	0.70	0.65	

		Butyrate mM		
In vitro Sulfite	**4 HR**	**8 HR**	**24 HR**	**SEM**
NO	8	10	12	0.5
YES	9	11	13	0.5
P- value	0.23	0.23	0.12	

		A:P		
In vitro Sulfite	**4 HR**	**8 HR**	**24 HR**	**SEM**
NO	2.42[a]	2.42[a]	2.35[a]	0.097
YES	2.22[b]	2.23[b]	2.18[b]	0.097
P- value	0.0003	0.0006	0.002	

		Total VFA		
In vitro Sulfite	**4 HR**	**8 HR**	**24 HR**	**SEM**
NO	78	89	98	5.4
YES	74	84	96	5.4
P- value	0.43	0.33	0.79	

[a-b] Means within a column not connected by the same letter are significantly different ($P < 0.05$).

Table 6.9: Effects of donor diet and added buffer-sulfite combinations on in vitro cumulative H_2, CH_4, and CO_2 production at 4, 8, and 24 h of incubation.

		Cumulative H_2 mmol			
Donor diet	**In vitro sulfite**	**4 HR**	**8 HR**	**24 HR**	**SEM**
Bicarb	NO	0.002	0.006	0.003	0.0059
Bicarb	YES	0.021	0.052	0.014	0.0068
Bicarb + Sulfite	NO	0.003	0.004	0.003	0.0068
Bicarb + Sulfite	YES	0.017	0.008	0.005	0.0059
Acetate	NO	0.009	0.03	0.012	0.0059
Acetate	YES	0.012	0.038	0.006	0.0065
Acetate + Sulfite	NO	0.004	0.005	0.006	0.0059
Acetate + Sulfite	YES	0.038	0.01	0.001	0.0059
P-value		0.069	0.078	0.112	
		Cumulative CH_4 mmol			
Donor diet	**In vitro sulfite**	**4 HR**	**8 HR**	**24 HR**	**SEM**
Bicarb	NO	0.048^{abd}	0.24	0.472	0.052
Bicarb	YES	-0.008^{ce}	0.059	0.203	0.0575
Bicarb + Sulfite	NO	0.046^{abcde}	0.27	0.505	0.0526
Bicarb + Sulfite	YES	0.021^{bcde}	0.194	0.494	0.0521
Acetate	NO	0.055^{abc}	0.224	0.577	0.052
Acetate	YES	0.013^{de}	0.161	0.421	0.054
Acetate + Sulfite	NO	0.088^{a}	0.322	0.693	0.052
Acetate + Sulfite	YES	0.021^{bcde}	0.248	0.595	0.052
P-value		0.03	0.32	0.24	
		Cumulative CO_2 mmol			
Donor diet	**In vitro sulfite**	**4 HR**	**8 HR**	**24 HR**	**SEM**
Bicarb	NO	2.454	2.641^{ab}	3.414	0.4152
Bicarb	YES	2.049	2.207^{ab}	3.651	0.4532
Bicarb + Sulfite	NO	2.123	2.536^{ab}	3.624	0.4205
Bicarb + Sulfite	YES	2.424	2.832^{ab}	4.167	0.4152
Acetate	NO	2.629	3.039^{a}	4.233	0.4152
Acetate	YES	1.916	1.779^{b}	3.605	0.4504
Acetate + Sulfite	NO	3.448	3.761^{a}	5.307	0.4152
Acetate + Sulfite	YES	2.671	3.188^{ab}	4.491	0.4152
P-value		0.32	0.06	0.32	

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

Table 6.10: Effects of donor diet and added buffer-sulfite combinations on in vitro VFA concentrations and acetate:propionate ratio at 4, 8, and 24 h of incubation.

		Acetate mM			
Donor Diet	**In vitro Sulfite**	**4 HR**	**8 HR**	**24 HR**	**SEM**
Bicarb	NO	40.3	49.9	50.9	6.23
Bicarb	YES	40.4	37.4	41.7	6.23
Bicarb + Sulfite	NO	45.7	49.9	59.6	6.23
Bicarb + Sulfite	YES	44.6	51	58.5	6.23
Acetate	NO	52.6	59.6	69.3	6.23
Acetate	YES	46.3	53	61.8	6.23
Acetate + Sulfite	NO	54.7	59.3	60.9	6.23
Acetate + Sulfite	YES	46.5	58.3	64.2	6.23
P-value		0.47	0.24	0.44	

		Propionate			
Donor Diet	**In vitro Sulfite**	**4 HR**	**8 HR**	**24 HR**	**SEM**
Bicarb	NO	17.2	22.8	22.1	2.97
Bicarb	YES	20.3	17.8	21.2	2.97
Bicarb + Sulfite	NO	18.8	20.3	25.3	2.97
Bicarb + Sulfite	YES	19.6	22.5	26.5	2.97
Acetate	NO	21.8	24.3	29.1	2.97
Acetate	YES	19.8	23	27.9	2.97
Acetate + Sulfite	NO	25.9	27.1	27.2	2.97
Acetate + Sulfite	YES	23.7	28.9	31.6	2.97
P-value		0.44	0.29	0.69	

Butyrate mM					
Donor Diet	**In vitro Sulfite**	**4 HR**	**8 HR**	**24 HR**	**SEM**
Bicarb	NO	6.5	8.7	9	1.52
Bicarb	YES	8.4	8.1	9.6	1.52
Bicarb + Sulfite	NO	8.2	9	11.5	1.52
Bicarb + Sulfite	YES	9.2	11.1	12.8	1.52
Acetate	NO	7.8	10.8	13	1.52
Acetate	YES	8.4	11	13.2	1.52
Acetate + Sulfite	NO	11.1	12.5	12.9	1.52
Acetate + Sulfite	YES	10.8	14.1	15.9	1.52
P-value		0.71	0.51	0.65	

A:P					
Donor Diet	**In vitro Sulfite**	**4 HR**	**8 HR**	**24 HR**	**SEM**
Acetate	NO	2.5	2.5	2.4	0.14
Acetate	YES	2.3	2.3	2.2	0.14
Acetate + Sulfite	NO	2.2	2.2	2.2	0.14
Acetate + Sulfite	YES	2	2	2	0.14
Bicarb	NO	2.6	2.5	2.4	0.14
Bicarb	YES	2.2	2.2	2.1	0.14
Bicarb + Sulfite	NO	2.5	2.5	2.4	0.14
Bicarb + Sulfite	YES	2.4	2.4	2.3	0.14
P-value		0.68	0.75	0.65	

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