Effects of bismuth subsalicylate and beta extract of hops (*Humulus lupulus*) on in vitro fermentation with ruminal microbes

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ABSTRACT

Symbiosis between microbes and ruminants gives the animal a unique ability to digest fiber and transform it into meat, milk, power and other useful products. Manipulation of rumen ecology with selective antimicrobial compounds can have beneficial effects by altering microbial output, allowing the animal to achieve greater levels of production per unit of input. Two experiments were conducted to determine effects of antimicrobial compounds on in vitro fermentation with ruminal microbes in continuous culture. Inclusion of bismuth subsalicylate decreased ($P < 0.05$) organic matter digestion, volatile fatty acid production and had negative influences on nitrogen and fatty acid metabolism. Results indicate that bismuth subsalicylate at 0.5% of diet dry matter was detrimental to overall fermentation with rumen microbes, and lower dosage levels should be investigated. In experiment 2, beta extract from the hop plant (*Humulus lupulus*) was administered to continuous culture fermenters at 0, 600, 1200 or 1800 mg of beta acids / kg of dry matter. Inclusion of beta extract did not affect ($P > 0.05$) ingredient digestion, volatile fatty acid production or nitrogen metabolism. Beta extract tended ($P = 0.09$) to increase culture pH, however effects were modest and lower than biologically relevant values. Further research investigating the adaptation of microbial populations to hop beta extract was recommended.

Keywords: continuous culture, fermentation, rumen, bismuth subsalicylate, hops, selective antibiotics

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LIST OF ABBREVIATIONS

AA: Amino acid

ATP: Adenosine triphosphate

ADF: Acid detergent fiber

BCVFA: Branched-chain volatile fatty acids

BE: Beta-extract of hops

BSS: Bismuth subsalicylate

CP: Crude protein

DIP: Dietary insoluble protein

DM: Dry matter

EMPS: Efficiency of microbial protein synthesis

H_2S: Hydrogen sulfide

K_d: Rate of degradation

K_p: Rate of passage

NDF: Neutral detergent fiber

NH_3-N: Ammonia nitrogen

NPN: Non-ammonia nitrogen

OM: Organic matter

PEM: Polioencephalomalacia

PSM: Plant secondary metabolite

VFA: Volatile fatty acid

LITERATURE REVIEW

Manipulation of rumen fermentation with selective antimicrobial compounds

INTRODUCTION

In ruminants, symbiosis between microorganisms and host is a complex and unique relationship. The pre-gastric location and unique physiologic aspects of the rumen allow the animal to utilize substrate not readily digested by non-ruminants. Due to limited oxygen and readily available nitrogen sources, strictly anaerobic bacteria capable of digesting complex carbohydrates (such as cellulose) are able to flourish in the rumen. Cellulose is the most abundant organic molecule on earth and the ability to transform this product into useful material is advantageous to the host from an evolutionary point of view. While non-ruminants generally have greater overall efficiency of production (Black, 1970), ruminant animals are able to more efficiency digest cellulose (Uden and Van Soest, 1972), allowing them to utilize feedstuffs with greater concentrations of fiber.

Humans have recognized the importance of cellulosic material for a long time. Early dwellings and tools were likely made of wood and heat was largely provided via combustion of cellulosic material. When ruminants were domesticated 10,000 years ago, their ability to convert grasses and other fibrous plants into usable products (meat, milk, hide, bone, and draft power) was important for the transformation from hunter-gatherer to agrarian society (Hatziminaoglou and Boyazoglu, 2004). Many thousands of years later, ruminant animals continue to support societies in a similar fashion. More recently, humans have sought to remove the animal from this equation, instead seeking an industrial process to transform cellulose to usable energy forms. It has been identified

that microbial populations can ferment carbohydrate substrate into compounds such as ethanol and methane. Hungate (1950) summarized the concept of an efficient industrial process for cellulose fermentation as an automated system with consistent inflow of substrate and sufficient removal of end-products, permitting high levels of cellulose fermentation. He pointed out that while an efficient industrial process is still far from reality, nature has already accomplished this task in the ruminant animal; an individual fermentation unit that collects substrate, facilitates removal of end products, generates usable products for human consumption and even reproduces itself.

Individuals involved in ruminant production and nutrition recognize benefits of the ruminant animal and microorganism symbiosis. Improvements in animal husbandry, selective breeding, targeted feeding and use of technology has helped us improve productive efficiency. The current generation of dairy cattle produce almost three times as much milk, and grow twice as fast as animals from 50 years ago (Capper et al., 2009). Further advances in efficiency will likely come through a more thorough understanding of rumen ecology and its role in individual animal performance (Weimer, 1998).

The first section of this review will provide a brief description of rumen function and ecology, followed by an overview of ruminal degradation of the two largest components of feedstuffs; carbohydrates and proteins. The second section will be dedicated to the concept of an ‘ideal’ fermentation and the various methods used to manipulate rumen fermentation. This will be followed by specific examples including the use of several compounds known to exhibit selective inhibition of rumen microorganisms.

RUMEN FERMENTATION

Rumen Digestive Physiology

The four chambered stomach of the ruminant animal is separated into the rumen, reticulum, omasum, and abomasum. The rumen comprises of the vast majority of the pre-gastric tract volume and supports a diverse microbial population. The main function of the rumen is to provide a mostly anaerobic environment capable of selectively retaining substrate for microbial and mechanical particle size reduction. This is largely achieved through the process of rumination where dietary substrate is degraded to smaller particle sizes through mastication and microbial degradation. The act of mastication action also stimulates saliva production, which aids in buffering and flow of rumen contents. The rumen epithelial wall is uniquely adapted for absorption of microbial end products. Volatile fatty acids (VFA) in their undissociated forms are passively absorbed via a bicarbonate exchange system with plasma (Dijkstra, 1994). In the reticulum, careful sorting of feed particles occurs. Here, the omasal orifice allows for selective passage of liquids and smaller particles, while retaining larger particles for further physical and microbial degradation in the rumen (Van Soest, 1994).

After passage from the reticulo-rumen, digesta enters the omasum, where leaf-like projections aid in the absorption of water and likely a large amount of VFA. Digesta then enters the abomasum, which is a glandular compartment analogous to the gastric stomach of non-ruminant animals. In the abomasum, hydrochloric acid and pepsin secretions continue degradation of feed particles. At this point, microbial cells are lysed and cell components are available for digestion and absorption in the lower tract.

Rumen Microbial Ecology

The rumen hosts a large and diverse population of microorganisms that are generally divided into bacteria, archaea, protozoa and fungi. Initial efforts to describe microbial populations in the rumen were based on morphologic traits, substrate utilization, end-product accumulation and simple biochemical tests (Hungate, 1966). These efforts, while extremely important, lacked any true genetic component and thus provided limited insight into genetic diversity of rumen microbial ecology. With the advent of DNA and RNA based technologies, genetic relationships could be elucidated (Whitford et al., 1998).

From a nutritional point of view, general categorization of the various microbial species is based on type of substrate utilized and/or end-products generated. Fiber, non-fiber, proteolytic and methanogenic classifications are most commonly found in the literature. In some instances, grouping is based on crude isolation technique, as is the case with protozoa, liquid associated bacteria (LAB) and solid associated bacteria (SAB) fractions. Liquid associated bacteria are often considered to be non-structural carbohydrate digesters, while SAB are typically considered to be fibrolytic organisms, although this separation is not definitive. It is likely that the LAB portion also contains a significant number of fibrolytic organisms that are simply not attached to substrate at the time of separation (Russell, 2002). In this review, specific species will be mentioned only when appropriate, otherwise general categories will be used. Table 1.1 depicts these general categories, along with some example organisms. Gram staining characteristics are not included in this table because some individual species exhibit variable staining tendencies based on cultivation method (Beveridge, 2001).

Because of their size and complexity, protozoa are the most conspicuous of the rumen microbes under a light microscope. Their role in the rumen is complex and not fully understood (Williams and Coleman, 1997). When animals are kept in isolation from birth, defaunation can occur, thus demonstrating that protozoa are not essential to rumen function (Bird and Leng, 1984). Defaunation can be defined as elimination of all protozoa in the rumen. The beneficial or detrimental role of protozoa also seems to depend on diet. Detrimental effects may be realized in low rumen N situations where sequestration of engulfed bacteria can limit microbial output (Newbold and Hillman, 1990). Some methanogenic archaea are associated closely with ciliated protozoa (Sharp et al., 1998) and high populations of methanogens can contribute to energy loss through methane production. Potentially beneficial aspects of rumen protozoa include engulfment of starch granules which sequesters them from rapid digestion to fermentation acids and modulates fluctuations in rumen pH. Coleman (1992) demonstrated a tremendous ability of *Entodinium sp.* to take up starch while Towne et al. (1990) and Nagaraja et al. (1992) noted beneficial modulating effects on fermentation pH when ciliated protozoa were present in cattle fed feedlot diets.

The role of fungi is not completely understood (Williams and Newbold, 1990). Fungal sporangium attachment to fiber and penetration of rhizoids into the plant cuticle has been postulated to aid in the access of fibrolytic organisms into the fiber matrix (Tomme et al., 1995). Fungal populations also express cellulases of their own but viability and function of these enzymes in the rumen is uncertain because enzymes may be rapidly degraded by proteolytic enzymes in the extracellular space (Nsereko et al., 2000).

Rumen Carbohydrate Metabolism

Many types of carbohydrates are present in ruminant diets. Carbohydrate classification is largely dependent on focus of the classifier. Plant physiologists typically emphasize biosynthesis, while animal nutritionists emphasize biodegradation (Van Soest, 1994). In this review, major nutritionally relevant categories will be briefly discussed in relation to their relative rate of degradation in the rumen. The three general categories are simple sugars (mono and disaccharides), plant energy storage compounds (starch, fructans) and structural carbohydrates (principally pectin, hemicellulose, and cellulose). The latter category forms the main bulk of forages in ruminant nutrition and is a subject of great interest.

Nutritional relevance of each carbohydrate category has led to the creation of various fractionation schemes for carbohydrate, especially in regard to the fiber portion. The generally accepted scheme currently in use is the detergent fiber system, developed by Van Soest et al. (1991). Other proposed systems have attempted to isolate more chemically uniform feed fractions and relate these fractions to specific animal performance (Prosky et al., 1988; Theander et al., 1995). Lucas (1964) developed a statistical test to determine uniformity of degradation of nutrients in wide ranges of feeding situations and levels of intake. Application of the Lucas test indicates that the current alternatives to the detergent fiber system do not represent nutritionally significant or uniform fractions (Van Soest, 1967).

Ruminal digestion of carbohydrate is initiated by extracellular hydrolysis of polysaccharide to form smaller saccharides (Martin, 1994). Intracellular fermentation of

saccharides (mostly hexose) forms pyruvate as an intermediate product, which is then metabolized to VFA (Russell and Hespell, 1981). Due to the general lack of oxygen for final electron disposal, oxidation reactions must be accompanied by a simultaneous reduction (redox). Therefore, ruminal fermentation of carbohydrate is often described as an exergonic process that results in accumulation of incompletely oxidized end products (Van Soest, 1994; Russell, 2002). Figure 1.1 depicts the common pathways of carbohydrate utilization in the rumen.

Extent and rate of digestion of more complex polysaccharides are important measures to nutritionists. In grain seeds, structure of the kernels reflects biological function of the seed, eg. protection and transportation of the embryo to a fertile place for germination. The endosperm contains most of the starch for the germinating seed which is protected by storage proteins. An example would be in corn where starch granules are encapsulated in a protein matrix (Pomeranz et al., 1984). These prolamin proteins resist enzymatic degradation, limiting rate of hydration and digestion of starch (Lopes et al., 2009).

Structural carbohydrate digestion is also largely influenced by physical factors such as the complex matrix of cellulose, phenolic compounds and some proteins bound in the plant cell wall. Structure of the cell wall and degree of cross-linking between phenolic compounds and hemicellulose vary according to type of plant (Chesson and Forsberg, 1997). Legumes exhibit a higher rate of digestion compared with grasses, however extent of digestion may be lower, depending on many factors including growing environment, variety and maturity (Van Soest, 1994). Neutral detergent fiber from plant parts not associated with structural components demonstrates highly fermentable characteristics

resulting from a lack of vascular tissue. Beet pulp and soybean hulls provide highly fermentable NDF (Hoover, 1986).

Ultimately, rate and extent of carbohydrate degradability is considered to be a major factor in determining intake and thus energy limitations. An increase in energy density of a ration by NDF replacement with easily fermentable grains has serious limitations associated with ruminal acidosis (Owens et al., 1998). Scientific evidence and practical feeding experiences have shown that rumination must be maintained to support buffer and saliva flow to the rumen (Mertens, 1997). Optimal carbohydrate digestion can be achieved by balancing rumen available carbohydrate and roughage to support proper rumination and thus buffering and dilution of fermentation acids.

Rumen Protein Metabolism

Nitrogen is an important element in ruminant diets. Dietary nitrogen is introduced through proteins, peptides and amino acids along with non-protein nitrogen (NPN) compounds such as ammonia and urea. Nitrogen is also supplied via recycled N in the form of urea in the saliva and N secretions associated with rumen epithelial cell turnover. Despite different forms of dietary N, feedstuffs are typically valued by their crude protein (CP) content. While the traditional CP = N x 6.25 equation is convenient, animals do not have a CP requirement and the equation is not sensitive to the type of nitrogen compound provided in the diet. For example urea contains 46.6% nitrogen, while Lysine contains 15.34% nitrogen. Inadequacy of this system has long been understood and alternative systems to quantify metabolizable protein have been proposed and hotly debated (Burroughs et al., 1975; Satter, 1982; Fox et al., 1992; Wilkerson et al., 1993).

Animals require specific essential amino acids for maintenance, growth, and production. Similar to non-ruminants, lactating dairy cattle have limiting amino acids, most often lysine or methionine (Schwab et al., 1992; Rulquin et al., 1993). Targeted feeding of these amino acids in pure form is complicated by high activity of microbial deamination in the rumen. Studies using cannulated cows have shown that amino acid flow to the small intestine is largely unaffected by the amino acid profile of the original feed (Van't Klooster and Boekholt, 1972; Clark et al., 1992). This lack of effect of amino acid profile in the feed can be beneficial in diets with poor quality protein feeds if adequate levels of soluble nitrogen and fermentable carbohydrate are present to promote ruminal microbial protein synthesis (Firkins, 1996; Bach et al., 2005). However, this relatively stable AA content reaching the small intestine represents a loss when a diet high in RDP with a favorable amino acid profile is fed, because the original amino acid profile will be altered by the rumen microbes. Protection of certain amino acids, particularly lysine, is accomplished by incorporation in a saturated fat-amino acid matrix, encapsulation in a fat or polymer coating or a combination of both. If protection is adequately disrupted after the rumen, amino acids can be supplied to the small intestine for absorption (Schwab, 1996).

Rumen microorganisms are able to synthesize many of their own amino acids from nitrogen and carbon sources in the rumen (Allison, 1969; Argyle and Baldwin, 1989). The first step in dietary protein degradation is hydrolysis by membrane bound proteases (Brock et al., 1982). Resulting peptides and amino acids are then transported into the cell where they can be incorporated in microbial protein or further degraded to ammonia. Amino acid uptake by most growing rumen microbes is high (Argyle and Baldwin,

1989), but some species lack transport mechanisms to move excess amino acids into the extracellular medium. Therefore, export of nitrogen as ammonia is crucial to avoid cellular death (Tamminga, 1979; Russell et al., 1988). Ammonia has been recognized as an important nitrogen source for rumen microbes for many years (Lewis et al., 1957). In fact, Virtanen (1966) demonstrated that fermentation could be maintained when cows were fed a diet containing no protein. Satter and Slyter (1974) performed a series of in vitro and in vivo experiments on ammonia requirements by rumen microbes. Their data suggest that concentrations of 2 to 5 mg/dL of rumen fluid are needed to optimize microbial fermentation (Satter and Slyter, 1974; Satter and Roffler, 1975; Slyter et al., 1979). However, in feeding situations with excess RDP and soluble nitrogen sources, ammonia production can overwhelm microbial uptake of ammonia, leading to an accumulation of ammonia in the rumen (Bartley et al., 1976). Ammonia production can also increase when energy is the limiting factor, because some bacterial species can utilize carbon skeletons from deaminated amino acids for ATP production, albeit at lower energy efficiencies than carbohydrates (Krause and Russell, 1996).

Amino acid flow to the small intestine is the summation of dietary insoluble protein (DIP), microbial protein and intact pre-intestinal endogenous protein secretions. This net amino acid flow is commonly referred to as metabolizable protein (MP) and must cover the requirements of the animal. Researchers have sought to model outflow of MP and predict the amino acid profile of the pool. Many models assume a specific amino acid profile for the entire pool or various sub-divisions based on the source (feed or microbial origin) (O'Connor et al., 1993; Baldwin, 1995; Lescoat and Sauvant, 1995). Outputs from these models vary considerably, with the more mechanistic models providing greater

accuracy, but often at the expense of field applicability due to the need for specific inputs not available in most production settings (Bannink et al., 1997; Offner and Sauvant, 2004). Research has identified that the amino acid profile of microbial protein is close to ideal, therefore feeding strategies should first maximize microbial protein output, followed by inclusion of DIP and supplementation with any possible limiting amino acids to meet amino acid requirements of the animal.

Synchronization of Rumen Nitrogen and Available Carbohydrate

In general, research findings agree that synchronization of rumen carbohydrate and nitrogen availability from all sources is essential for optimal ruminal fermentation. Readily fermentable carbohydrate sources are more effective than fiber at promoting microbial uptake of degraded protein (Stern and Hoover, 1979). In vivo studies demonstrated that ruminal infusion of readily fermentable carbohydrate sources decreased NH_3-N concentration by increasing N use by rumen microbes (Casper and Schingoethe, 1989; Cameron et al., 1991). This decrease in NH_3-N concentration can be explained in two ways: 1) the increase in energy from carbohydrate metabolism allows for higher growth rates and more assimilation of ammonia, and 2) non-fibrolytic bacteria can incorporate amino acids and peptides directly, thereby reducing NH_3-N production (Russell, 2002). Fibrolytic bacteria depend on NH_3-N as a nitrogen source (Russell et al., 1992). These concepts have led to a recommendation to synchronize levels of rumen available carbohydrate and N, however production responses to synchronization of dietary fermentable N and carbohydrate substrate is difficult to accurately study because many types of bacteria utilize amino acids and carbohydrates as energy sources (Bach et al., 2005). In contrast, recent work found that asynchronous dietary supply of N may not

be detrimental. Reynolds and Kristensen (2008) demonstrated that oscillation of rumen available N levels (asynchronous supply) increased nitrogen retention, probably due to modulating effects of recycled nitrogen. Isotope studies have shown that recycled NH_3-N can account for 23 to 95% of microbial incorporated nitrogen (Nolan and Dobos, 2005; Firkins et al., 2007; Recktenwald, 2010). Thus, the N economy of the ruminant is largely dependent on the type of bacteria present as affected by diet, and quantity of rumen available N fed. Greater nitrogen recycling with low dietary rumen available N is an evolutionary advantage to the ruminant animal because increased flows of urea nitrogen allow for more steady supply of N to the microbial population.

MANIPULATION OF RUMEN FERMENTATION

Goal of Fermentation and Major Factors Determining Microbial Ecology

The ruminant animal is dependent on fermentation products for most of their nutrients. Assuming that 60% of dietary DM is digested in the rumen, high producing dairy cattle can produce in excess of 160 moles of VFA (3 liters), and approximately 3 kg of microbial protein per day (Russell, 2002). This unique aspect of the ruminant animal forces the producer to focus on maintaining a healthy, active fermentation. Because the rumen ecosystem is dynamic and diverse, it is no surprise that nutritionists have sought to alter fermentation to produce more beneficial end products per unit of input. Russell (2002) described the concept of an ideal fermentation and listed the key characteristics as:

1) **Rapid rates of fiber digestion**. Fiber is an important part of the ruminant diet, and provides much of the physical bulk of the diet. If the degradation rate is slow, food intake decreases.

2) **Rapid and efficient production of microbial protein**. Slower rates of microbial growth reduce efficiency of microbial yield because energy is diverted to maintenance and energy spilling.

3) **Limited ammonia accumulation**. Excess ruminal proteolysis results in loss of available amino acids and excess ammonia accumulation. The animal must convert absorbed ammonia to urea, which is an energy consuming process.

4) **Limited methane production**. Methane acts as a ruminal metabolic hydrogen sink, however energy contained in the bonds is unrecoverable by the animal. As much as 11% of feed energy can be lost through methane emission. Diverting the reducing equivalents from methane to propionate can improve energy efficiency.

5) **Optimal ratio of VFA**. Propionate is the major glucogenic precursor in ruminants, while acetate and butyrate cannot be used for glucose synthesis. If inadequate propionate is produced, the animal resorts to amino acids for glucogenic precursors.

6) **Limited lactate accumulation**. Lactic acid is a 10-fold stronger acid than VFAs. Lactate accumulation can lead to reductions in ruminal pH, fiber digestion and microbial protein synthesis and can even cause systemic acidosis.

7) **Fewer toxins**. Fermentation in the rumen can degrade dietary toxins, thus protecting the animal.

-Adapted from Russell (2002)

To achieve an optimal fermentation, nutritionists must consider the complex interactions of diet, animal behavior, environment and animal genetics (Van Soest, 1994; Weimer et al., 1999; Li et al., 2009). Composition and degradation kinetics of the diet is one of the largest external variables influencing the rumen microbial ecosystem. Rate of degradation (k_d) and rate of passage (k_p) through the digestive tract determine the portion of substrate escaping degradation, as illustrated by equation 1:

Equation 1: $R = \frac{k_p}{k_p + k_d}$

Where R is the indigestibility coefficient, k_d is rate of degradation and k_p is rate of passage from the digestion chamber. This form is preferable to the commonly used equation substituting k_d for k_p in the numerator, which expresses apparent digestibility of the feedstuff. Indigestibility coefficients are inherently more useful for mathematical applications, in particular the calculation of true partial digestion coefficients. When calculating true digestibility, use of digestion coefficients will cause metabolic contribution to be expressed in negative terms (Van Soest, 1994). Using this equation, it is easy to see how manipulating any one rate will affect extent of degradation. These important variables are largely determined by various physical and chemical aspects of the feed, as described in the previous section.

Once the larger effects of diet composition have been accounted for, nutritionists can manipulate rumen ecology using of small amounts of bioactive supplements. Dietary inclusion of specific compounds can promote or suppress certain groups of microorganisms and alter final microbial output. In this review, bioactive supplements will be divided into those that promote beneficial microbial populations (probiotics and prebiotics) and those that suppress specific microbial populations (selective antibiotics). More emphasis will be placed on the latter group and examples will be provided.

Selective Promotion of Rumen Microorganisms

Use of probiotics and prebiotics to manipulate fermentation has received greater attention in recent years. Probiotics are feed supplements that contain live cultures of beneficial microorganisms including yeasts, fungi, and bacteria. Conceptually, these

microorganisms can produce beneficial end products such as VFA and microbial protein, and compete with detrimental or pathogenic organisms for substrates and attachment sites (Newbold, 1997; Huber, 1997; Chaucheyras-Durand and Durand, 2010). Fermentation responses to probiotics have been inconsistent. Review papers and meta-analysis of yeast supplementation studies tend to agree that supplementation appears to be beneficial, but effects are likely dependent on many different factors, including diet composition, preparation of yeast supplement and even breed of cow (Desnoyers et al., 2009; Thrune et al., 2009; Chaucheyras-Durand and Durand, 2010). The mode of action of fungal supplements (typically *Aspergillus oryzae*) is not completely understood, but probably follows a similar role to native fungal populations in the rumen, as discussed in the previous section on rumen fungi.

Prebiotics were initially defined by Gibson and Roberfroid (1995) in relation to human nutrition. They defined prebiotics as "a non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon." Based on this definition, much of the work with prebiotics has been restricted to non-ruminants (Patterson and Burkholder, 2003; Mountzouris, 2007). In ruminants, microbial populations degrade prebiotics in the rumen with little influence on specific populations or net microbial output (Callaway et al., 2008).

Inhibition of Rumen Microorganisms

Antimicrobial activity of many compounds is typically determined initially using an in-vitro screening test, where the main focus addresses the minimum inhibitory

concentration (MIC) (Lambert and Pearson, 2000). The MIC technique allows for a large number of compounds to be identified, however information regarding the specific mode of action is rarely available (Burt, 2004). Use of in vitro screening alone can lead to some compounds being overlooked if the organism or medium of interest is not similar to the conditions of the in vitro test. Although specific modes of action are lacking, researchers tend to agree that the cell membrane is central to many antimicrobial characteristics (Burt, 2004). Cell membranes are typically affected by transport of biologically important molecules across the membrane in contrast to the goals of the cell or through total disruption of the membrane, leading to catastrophic changes in membrane fluidity and/or cell lyses and death.

An appropriate example of the first mode of action is the ionophore. These are hydrophobic molecules that freely dissociate into cellular membranes. Ionophores have a monovalent functional group capable of binding a proton, along with an ion binding site, where monovalent (Na^{+} and K^{+}) or divalent cations (Ca^{++} and Mg^{++}) are bound. Some ionophores can bind several types of cations, although many have selectivity for specific ions (Pressman, 1976). Once incorporated into a membrane, the molecule can effectively 'shuttle' protons across the membrane if the appropriate ion gradient is present. The direction of ion and proton movement is dictated by relative concentrations of the ion gradient in accordance with the Nerst equation (Hegarty and Gerdes, 1999). Many microorganisms maintain a higher concentration of potassium inside their cells and they expel sodium and protons. When treated with an ionophore, sodium and protons can enter the cell, resulting in a net increase of protons and a decline in intracellular pH. Cells then utilize membrane bound ATPase in an attempt to re-establish the proton motive force.

ATP levels quickly decline and the cell reaches a de-energized state that suppresses growth (Russell, 2002). Suppression of growth is typically referred to as bacteriostasis, as the cell is not actually killed in the process.

The secondary mode of action of bioactive compounds is centered on membrane disruption leading to cell suppression or death. Disruption typically starts with a compound forming bonds with specific elements of the lipid bi-layer, such as cholesterol or membrane bound proteins (Lohner and Blondelle, 2005). These bonds change the fluidity of the membrane and may limit the function of membrane bound proteins important to metabolism and growth (Keukens et al., 1995). The cell is then unable to conduct normal cell processes or adapt to changing environmental conditions. Some potent bactericidal compounds will disrupt the membrane in such a way causing rupture, lyses and cell death. Plant saponins are believed to work in this fashion (Makkar et al., 1995; Wang et al., 1998).

Selective Inhibition and the Issue of Adaptation

Efficacy of many antimicrobial compounds is not typically a broad spectrum phenomenon. In the case of ionophores, Gram-negative bacteria exhibit more resistance than cells that lack an outer protective membrane. Selective bacteriostasis can result in inhibition of certain microbial populations, shifting net microbial output to more beneficial end products such as propionate, the major glucogenic precursor in ruminants (Nagaraja et al., 1997). Bacteriostasis selectivity also limits the use of ionophores as effective antibiotics in human medicine, thus reducing the concern associated with antibiotic resistance. A broad spectrum antibiotic would have limited use in ruminant

production because overall depression of microbial activity in the rumen is not typically beneficial to animal growth and production.

The selective nature of ionophores is not always clear cut with Gram-positive cells. Some studies have shown that Gram-negative bacteria can be equally sensitive, while some Gram-positive cells exhibit low sensitivities to ionophores (Chen and Wolin, 1979). Pure culture studies have shown that ionophore resistance (specifically to monensin) is largely mediated by the presence of carbohydrates on the outer surface of the cells, as is the case with monensin-resistant *P. bryantii* B14 (Callaway and Russell, 1999). Bacteria can build these carbohydrates quickly and resistance is present even when two separate ionophores are alternated in a feeding regime (cross-resistance) (Newbold et al., 1992; Nikaido, 1998).

Naturally Occurring Selective Antimicrobial Compounds

Many of the naturally occurring microbiologically active compounds are plant secondary metabolites (PSM). These compounds are diverse and have broad reaching pharmacologic, industrial and agricultural uses. Many drug and animal nutrition companies routinely screen PSM to develop new products. The structures of over 200,000 PSM have been identified (Hartmann et al., 2009) and most of these compounds are terpenes, phenols and alkaloids (Bodas et al., 2012).

Terpenes are classified by the number of isoprene units. Monoisoprenes consist of many essential oils with a variety of radical groups, such as alcohols, aldehydes, ketones peroxides. Diterpenes are compounds such as phytol, tocopherol and retinol. Triterpenes are perhaps one of the most studied sub-categories, as they contain glycosides,

compounds that are well known for their application in pharmacology (Bodas et al., 2012). Saponins also fall into this category and have been shown to have moderate effects on rumen fermentations (Calsamiglia et al., 2007). Phenols are an extremely diverse category containing cinnamic acids, aflatoxins, lignins, condensed tannins and tetracyclines. Alkaloids are N containing compounds that are often extremely toxic or teratogenic to other organisms (Panter et al., 1999). Atropine, nicotine, codeine, morphine, lupinine and strychnine are examples of alkaloid compounds commonly found in plants.

Effects on Microbial Fermentation

Certain PSM are well recognized to work as selective antimicrobial agents against bacteria, protozoa and fungi (Bodas et al., 2012). The mode of action of glycoalkaloids was proposed by Keukens et al. (1995) and is shown in Figure 1.2. Bioactive compounds bind irreversibly to cholesterol in the lipid bilayer. Cholesterol then migrates across the membrane to bind to more glycoalkaloids which changes uniformity of the layer and allows for creation of lipid micelles from the cell membrane, forming a ring with the hydrophobic moieties combined with cholesterol. Membrane components are then removed from the cell, resulting in total membrane disruption.

Effects of saponins from *Yucca shidigera* on protozoa activity and alfalfa degradation have been well documented in vitro (Lu and Jorgensen, 1987; Wang et al., 2000). Hristov et al. (1999) observed antiprotozoal effects of saponins in heifers, although it is not well understood if all protozoa species are equally sensitive to these specific PSM (Patra and Saxena, 2009). Effects of PSM on ruminal incubations are well

documented and more comprehensive reviews are available (Wallace et al., 2002; Calsamiglia et al., 2007; Bodas et al., 2012). Various PSM are thought to alter the microbial profile by suppressing protozoa and Gram-positive bacteria (usually acetate producers) in such a way to allow for more favorable accumulation of propionate in the rumen (Patra and Saxena, 2009). Similar to monensin, these effects seem to be pH dependent, with more pronounced results seen at a rumen pH < 6 (Hristov et al., 1999; Lila et al., 2003; Devant et al., 2007; Hart et al., 2008).

The remainder of this review will focus on specific naturally-occurring compounds thought to have selective antimicrobial effects in the rumen, namely bismuth subsalicylate (BSS) and the various bioactive compounds of the hop plant. A brief background will be provided for each, followed by non-rumen and rumen applications of the specific compounds.

Bismuth Subsalicylate

Manipulation of rumen fermentation can reduce accumulation of detrimental end products. In the rumen, dietary sulfate is utilized as an electron acceptor by specific populations of microorganisms, resulting in a reduction to hydrogen sulfide (H_2S). *Desulfobrivio* and *Desulphotomaculum* are two such organisms capable of producing H_2S (Howard and Hungate, 1976). Excess dietary sulfur can lead to an accumulation of H_2S in the rumen. An increase in ruminal H_2S is associated with several undesirable conditions including decreased DM intake, growth performance and carcass quality (Lonergan et al., 2001) and an increase in sulfur associated dietary polioencephalomalacia incidence (Gould, 1998). Vanness et al. (2009) concluded that low ruminal pH conditions

associated with low dietary roughage favored reduction of sulfate to H_2S which is a concern in feedlot cattle fed high levels of distiller's grains, where ruminal pH is low and dietary sulfur is high. Typically, H_2S is absorbed through the rumen wall and detoxified in the liver before circulation into the blood stream. However, accumulation and eructation of gaseous H_2S can lead to re-inhalation by the animal, thereby bypassing hepatic detoxification. Hydrogen sulfide absorbed through the lungs can gain access to the blood stream and lead to necrosis of the cerebral cortex in acute cases and a decrease in cattle performance in subclinical cases (Gould, 1998).

Many different methods have been evaluated to manage H_2S accumulation in feedlot cattle. The principle focus has been on diet manipulation, ranging from varying roughage content (Vanness et al., 2009) to inclusion of thiamine, iron, or other minerals in an effort to bind sulfur compounds in the rumen (Felix and Loerch, 2011). Results from these approaches are inconsistent and nutritionists often revert to a recommendation that inclusion of high sulfur distiller's grains in the diet be limited (Kelzer et al., 2010a; Kelzer et al., 2010b).

Microbial reduction of sulfate is not only limited to ruminal fermentations. Fermentation of residual food and metabolic waste in the lower tract of humans also forms H_2S. Hydrogen sulfide has been associated with pathogenesis of ulcerative colitis, a risk factor for colorectal cancer (Rowan et al., 2009). Mitigation of H_2S toxicity is largely accomplished by decreasing dietary sulfur levels and providing compounds that bind sulfur compounds, limiting reduction to H_2S. One of these compounds is bismuth subsalicylate (BSS) which is commonly found in products such as Pepto Bismol® and De-Nol® (Suarez et al., 1998; Burford et al., 2002).

Use of bismuth compounds in cattle has been limited. In an in vitro screening test by Willard and Kodras (1967), anti-protozoal effects were observed with two bismuth preparations of arsenic and carbonate. Lower tract effects of BSS have shown positive results in mitigating calf diarrhea (Roussel and Brumbaugh, 1991; Constable, 2009). Veterinary grade BSS products are available and labeled for use in adult cattle. Rumen availability of these suspensions is not known because they are often provided in a slurry form designed to deliver BSS to the lower tract.

The only known research of BSS as a ruminal fermentation modifier was performed at the University of Minnesota by Moreno (2012). In vitro batch and continuous culture fermenters were used to evaluate effects of various levels of BSS on fermentation dynamics and H_2S accumulation. In the batch culture experiment, diets contained BSS at 0.5, 1, 2, and 4% of diet DM. Hydrogen sulfide content of the headspace was decreased ($P < 0.05$) at all levels of BSS inclusion with the 4 % of diet DM level resulting in a 99% decrease in H_2S concentration. However, reductions in VFA production and total gas accumulation were observed with BSS levels greater than 1% of diet DM indicating suppression of overall fermentation. Based on batch culture results, BSS inclusion at 1% of diet DM was administered to a dual-flow, continuous culture system. Bismuth subsalicylate inclusion markedly decreased H_2S production however total VFA concentration, CP degradation and efficiency of microbial protein synthesis also decreased with BSS addition. These observations indicated that BSS inclusion at 1% of diet DM was detrimental to overall fermentation. Titration studies are needed to determine optimal dose of BSS in fermentations with rumen microbes.

Hops and Hop Extracts

Hops (*Humulus lupulus*) have been used for centuries as a flavor, aroma and preservation agent in beer. For years, chemists and brewers alike were well aware of the antiseptic properties of hops because beers with higher hop inclusion exhibited fewer tendencies to spoil. With the rise of organic chemistry in the late nineteenth century, researchers began to experiment with several solvent extracts from cones of the female hop plant, with Lermer first identifying the crystalline form of the hop resin in 1863 (Ashurst 1967). Hayduck (1888) separated hop resin by extraction with several solvents and precipitation with lead acetate to isolate alpha and beta fractions of hop resins. Brown and Clubb (1913) validated this data by identifying that aqueous extract (referred to as "toxic water") reduced growth of unnamed Bacterium X. This finding led Walker at the Institute of Brewing of the United Kingdom to engage in long term research from 1922 to 1941, where he finally identified the specific active chemicals in the hop extracts. These naturally occurring plant compounds, namely the humulones (α-acids) and lupulones (β-acids) exhibited bacteriostatic properties in brewing applications. A far more comprehensive timeline can be found in more recently published reviews of hops in brewing applications (Verzele, 1986; Moir, 2000). Figure 1.3 shows the chemical structure of α, β and iso-α-acids.

The basis for antimicrobial activity of hop acids is the ability to disrupt normal cell membrane function. Trans-isohumulone has been shown to reduce uptake of leucine and cause slow leakage of any accumulated leucine from the cell (Simpson, 1993). The compound also effectively dissipates the transmembrane pH gradient, while maintaining transmembrane electric potential. Potentiometric studies demonstrated that trans-

isohumulone likely acts as an ionophore by interacting with divalent cations such as Mn^{++} (Sakamoto and Konings, 2003). Simpson and Smith (1992) similarly reported that protonophoric activity of the compound requires the presence of monovalent cations such as K^+, Na^+, or Rb^+, indicating selectivity of the compound for monovalent ions. Alternatively, Sakamoto et al. (2002) proposed that the weak acid properties of hop acids allow them to cross cellular membranes, and upon reaching the intracellular medium, the protonated compound dissociates resulting in net influx of protons. Non-ruminal in vitro studies of the antimicrobial activity of hops revealed that most hop compounds inhibit growth of Gram-positive bacteria (Shimwell, 1937; Schmalreck et al., 1975); a typical outcome of inhibition through membrane active molecules.

Non-brewing Applications of Hops

Effects of hops on non-brewing fermentations demonstrated varied results. Published studies have varied remarkably in how hop products were prepared, and include whole hops (ground, ensiled or whole cones), solvent extracts and CO_2 extracts. Due to the aerobic instability of beta acids, and possible confounding factors of other PSM and fermentable DM, studies involving whole hops should be interpreted with caution. Alpha and beta acid amounts were reduced when whole or ground hops are exposed to oxygen (Kaneda et al., 1990). Hops are also known to contain other PSM such as condensed tannins (CT), which have been shown to exhibit antimicrobial properties of their own (Makkar et al., 1995; Min et al., 2003). Investigation of antimicrobial properties of alpha and beta acids is very difficult because the compound must be evenly introduced to the fermentation with minimal aerobic exposure, limiting use of most conventional delivery systems such as incorporation with the fermentable substrate

(TMR). Possible confounding effects of CT and additional fermentable DM are difficult to control, and many studies involving whole or ground hops are plagued by confounded data (Krishna et al., 1986; Al-Mamun et al., 2011; Narvaez et al., 2012). Krishna et al. (1986) evaluated the possibility of using spent hops as an alternative feedstuff, resulting in high levels of fermentable substrate. This resulted in a high inclusion of CT (up to 5g CT per 100g of spent hops), which are known to inhibit rumen microorganism (Min et al., 2003). Narvaez et al. (2012) included ground hops with varied concentrations of alpha and beta acids, however did not include an adequate control containing similar levels of fermentable substrate or CT. Results from both these studies should be considered as the net effects of whole hop addition, not the specific antimicrobial activity of CT, alpha or beta acids.

Hops and Rumen Fermentation

In ruminal fermentations, extraction of alpha and beta acids by super critical CO_2 has allowed for more complete study of these specific compounds. Narvaez et al. (2011) observed in vitro decreases in CH_4 and NH_3-N accumulation and increases in molar proportion of propionate when 2400 mg β-acid/kg substrate was provided in batch culture. Schmidt and Nelson (2006) noted similar changes in VFA molar proportions on alfalfa substrate at much lower dosage (33 mg β-acid/kg substrate), however DM disappearance, gas production and total VFA production was also decreased, indicating general suppression of fermentation. Pure and co-culture studies have shown promising inhibition of hyper ammonia producing bacteria and increases in propionate production (Flythe, 2009; Flythe and Aiken, 2010).

In vivo work is more variable, probably due to difficulty in maintaining bioactivity of the compounds in aerobic feed storage. Wang et al., (2010) observed no effects on feedlot performance or carcass characteristics when hop pellets were fed to growing and finishing feedlot cattle. Wang and colleagues attributed the lack of positive results to a low level of feeding (40 and 80 mg beta acids/kg diet DM in a feedlot diet), and aerobic storage instability and heat associated with pelleting. Similarly, Drouillard et al. (2009) demonstrated little effect on rumen fermentation in feedlot cattle, however dosage was low at 30 mg of beta acids/kg of diet DM and extract was ruminally dosed once daily before feeding. Whole ensiled hop plants have been studied as an alternative roughage source in sheep and cattle (Zgajnar et al., 1987; Al-Mamun et al., 2011) however effects cannot be attributed to hop acids alone because their metabolic fate during ensiling has not been well defined. Neither study reported CT, alpha or beta acid concentration of the substrate.

Similar to other membrane active compounds, some species of bacteria exhibit resistance to bacteriostatic properties of hops. Sakamoto et al. (2002) tested effects of hop extract on membrane-bound H^+-ATPase activity in *Lactobacillis brevis* strains with and without previous exposure to hops. They demonstrated a four-fold increase in ATPase activity in naïve cells exposed to hop extracts. No changes in ATPase activity were reported in adapted cells, indicating that cells had developed a resistance mechanism that allowed the cell to maintain a proton motive force when confronted with an influx of protons from the ionophoric activities of the hop compound. Similar increases in ATPase activity were found in *Saccharomyces cerevisiae* cultures when cells were exposed to membrane active bacteriostatic compounds, suggesting that resistance mechanisms may

be conserved across genomes (Viegas et al., 1998) which is likely mediated by presence of a multi-drug resistant-like gene (Suzuki et al., 2002). Sakamoto and colleagues also altered medium pH from 4.4 to 6.8, and reported greatest ATPase activity at a pH of 5.6, while low activity was observed at pH 6.6 (Sakamoto et al, 2002) Results indicate d that resistance is greater at pH 5.6, and reduced as pH increased above 6. Batch culture incubations are highly buffered and pH does not often decline below 6.6, whereas in vivo and continuous culture often record pH values below 6.0. This phenomenon could partially explain differences in results between batch and in vivo experimentation with hop compounds.

SUMMARY

In conclusion, the degree to which various selective antimicrobial compounds influence fermentation is dependent on the large influence of diet, environment and level of exposure. Much of the in vitro work performed on specific compounds is likely dependent on conditions of the test. In vivo experimentation has often shown more variable effects and sustained alteration of the rumen ecosystem is often not attained with many selective antimicrobial compounds. Adaptation of rumen microbes to specific compounds is well documented and occurs to a certain degree in many situations. The success of a particular inhibiting compound is the result of its ability to create a sustained and significant shift in the rumen ecosystem, causing a beneficial change in net microbial output.

Table 1.1. Summary of general types of rumen bacteria

General Category	Abbreviation	Example Organisms
Fibrolytic or cellulolytic digesters	FC,CU, Fiber	*Ruminococcus sp., Fibriobacter succinogenes*
Non-structural carbohydrate digesters	NSC, NFC, Starch	*Selenomas ruminantium, Streptocuccus bovis, Provotella sp.*
Methanogens	CH_4	*Methanobrevibacter*
Hyper-ammonia producing bacteria	HAB, proteolytic	*Peptostreptococcus anaerobius, Clostridium sticklandii*

Figure 1.1. The metabolism of a hexose by various rumen fermentation pathways. Adapted from Russell (2002).

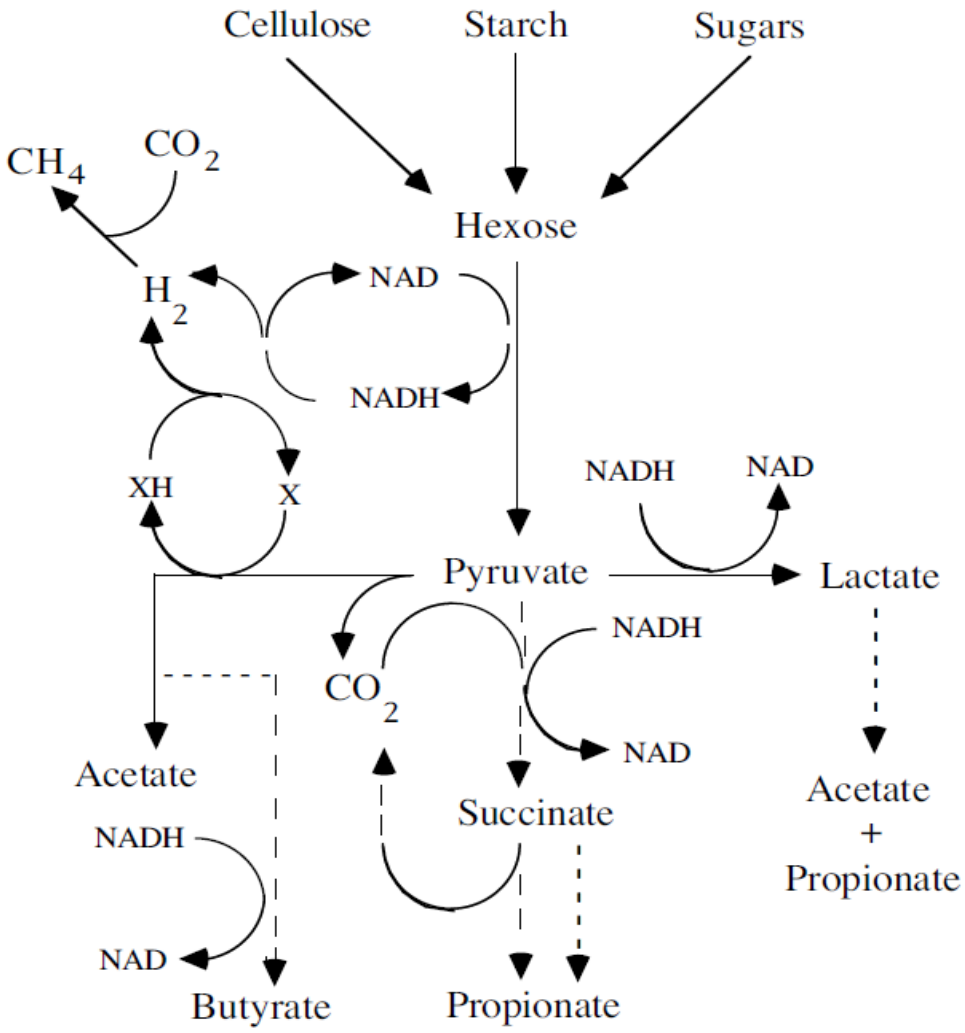


Figure 1.2. Hypothesized mode of action of glycoalkaloids in cellular membranes, where GA: glycoalkaloid, Chol.: Cholesterol, and PC: Phosphatidylcholine. Adapted from Keukens et al. (1995).

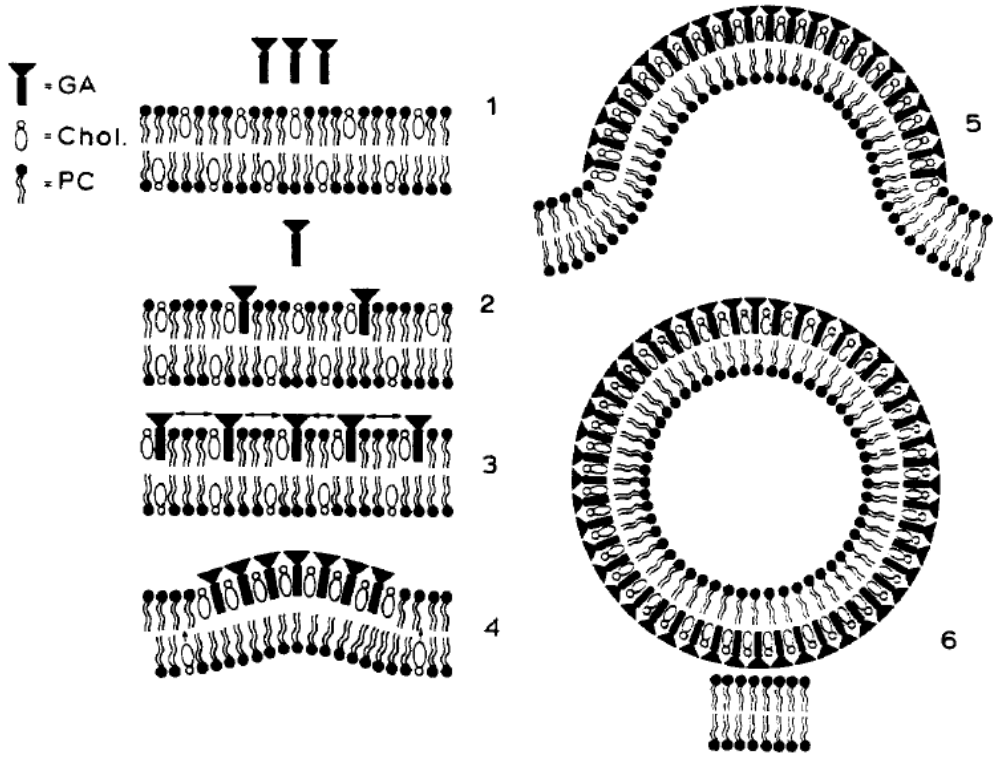
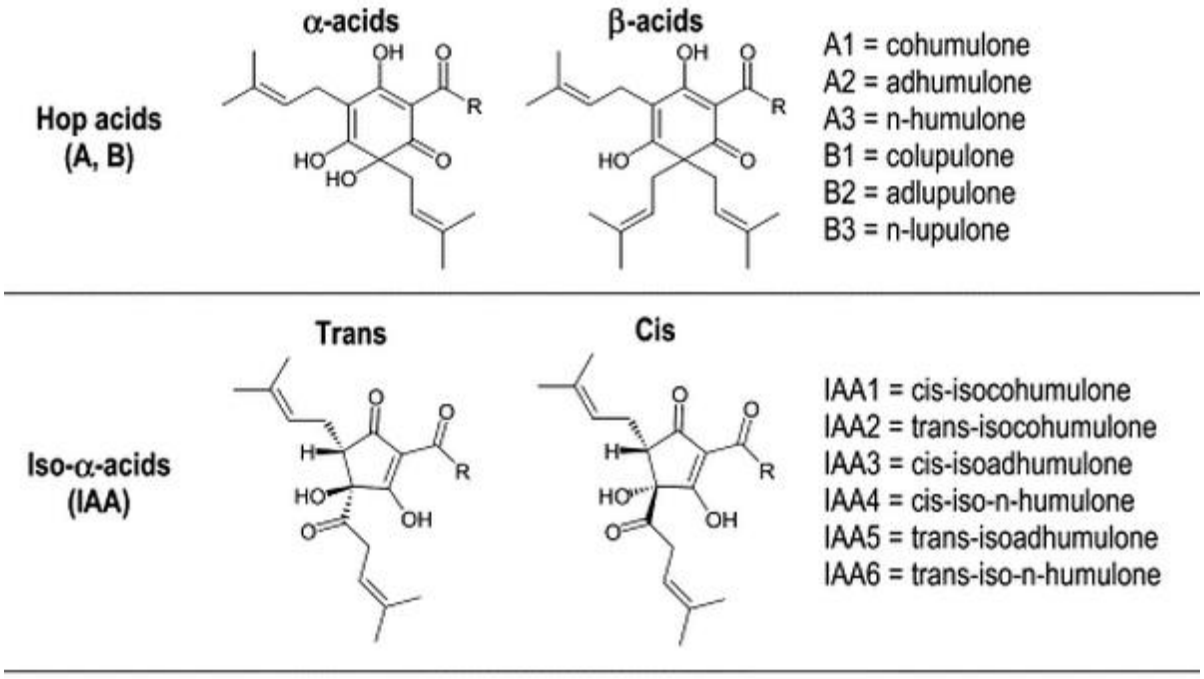


Figure 1.3. Chemical Structures of α, β, and iso-α-acids of the hop plant. Adapted from Vanhoenacker et al. (2004).



EXPERIMENT 1: Effects of bismuth subsalicylate and dietary sulfur level on fermentation by ruminal microbes in continuous culture

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Abstract: In ruminants, excess dietary sulfur is associated with a reduction in DM intake, poor feedlot performance and sulfur-associated polioencephalomalacia. Bismuth subsalicylate (BSS) has been shown to decrease hydrogen sulfide in vitro, however negative effects on fermentation were reported when BSS was included at 1% of diet DM. The objective of this experiment was to evaluate effects of BSS inclusion (0 or 0.5% of diet DM) and dietary sulfur (0.21 or 0.42% of diet DM) on microbial fermentation in continuous culture. Treatments were arranged in a 2 x 2 factorial design. Eight dual-flow continuous culture fermenters were used during 2 consecutive 10-d periods consisting of 7 d for stabilization followed by 3 d of sampling. A pelleted feedlot diet containing 39% dry rolled corn, 32% earlage, 21% wet distillers grains, 3.2% corn silage, 1.5% soybean meal, 0.6% urea and 2.7% mineral premix (DM basis) was provided as substrate for microbes at a rate of 75 g of DM/fermenter/d. Effluents from sampling days were composited by fermenter within period, resulting in 4 reps/treatment. BSS inclusion decreased ($P < 0.01$) true OM digestion, while no effects were observed for NDF and

ADF digestion. Total VFA concentrations, molar proportions of acetic, propionic, and branched-chained VFA decreased ($P < 0.01$) with BSS addition. The ratio of acetic to propionic acid and the molar proportion of butyric acid increased ($P < 0.01$) with BSS addition. In regard to nitrogen metabolism, BSS increased NH_3-N concentration, NH_3-N and dietary-N flows ($P < 0.01$), and decreased non-NH_3-N flow, microbial-N flow, CP degradation, and efficiency of microbial protein synthesis ($P < 0.01$). Inclusion of BSS increased mean, minimum, and maximum fermentation pH ($P < 0.01$). Fermentations were analyzed for hydrogen sulfide release; however no sulfide was detected in headspace samples. Amount of dietary sulfur and BSS inclusion influenced flows of amino acids and fatty acids from fermenters. Results from this experiment indicate that BSS included at 0.5% of diet DM has detrimental effects on in vitro rumen fermentation in continuous culture.

Keywords: bismuth subsalicylate, rumen, continuous culture, microbes, fermentation

INTRODUCTION

In ruminant animals, excess dietary sulfur is associated with several undesirable conditions including a reduction in DM intake, decreases in growth performance and carcass characteristics (Lonergan et al., 2001) and an increase in the incidence of sulfur associated polioencephalomalacia (PEM) (Gould, 1998). Dietary sulfur is reduced to hydrogen sulfide (H_2S) by specific bacteria such as *Desulfobrivio* and *Desulphotomaculum* (Russell, 2002). Vanness et al. (2009) concluded that low rumen pH conditions associated with low dietary roughage favored the reduction of sulfate to H_2S. This situation is a concern in feedlot cattle fed distiller's grains, where ruminal pH is low

and dietary sulfur is high. Hydrogen sulfide is normally absorbed through the rumen wall and de-toxified in the liver before circulation in the blood stream. However, accumulation and eructation of gaseous H_2S can lead to re-inhalation by the animal, thereby bypassing hepatic detoxification. Hydrogen sulfide that is absorbed through the lungs can gain direct access to the blood stream and lead to necrosis of the cerebral cortex in acute cases and poor growth performance in subclinical cases (Gould, 1998).

The only known research of BSS as a ruminal fermentation modifier was performed by Moreno (2012). In vitro batch and continuous culture fermenters were used to evaluate effects of several levels of BSS inclusion on fermentation dynamics and H_2S accumulation. In the batch culture experiment, diets contained BSS at 0.5, 1, 2 and 4% of diet DM. Hydrogen sulfide was decreased ($P < 0.05$) with all treatments and BSS at 4% of diet DM decreased H_2S concentration by 99%. However, detrimental effects on overall fermentation were observed when BSS exceeded 1% of diet DM. Based on results from batch culture, BSS inclusion at 1% of diet DM was evaluated in a dual-flow continuous culture system. Inclusion of BSS markedly reduced H_2S production, however total VFA concentration, CP degradation and efficiency of microbial protein synthesis were also decreased with BSS addition. These observations indicated that BSS inclusion at 1% of diet DM can be detrimental to overall fermentation by ruminal microbes.

The objective of this study was to determine effects of BSS inclusion at 0.5% of diet DM on in vitro fermentation with rumen microbes in diets with 0.21 or 0.42% sulfur. Primary emphasis was on BSS induced alterations of fermentation, leading to changes in microbial fermentation of major macronutrients. Secondary emphasis was on the

influence of the interaction of BSS with dietary sulfur level and impact on microbial fermentation.

MATERIALS AND METHODS

Animals and Collection of Rumen Fluid

The University of Minnesota Institutional Animal Care and Use Committee approved all animal use in this study. Two ruminally cannulated lactating dairy cows served as rumen fluid donors. The diet fed to donor cows was formulated to meet or exceed requirements of a Holstein cow producing 40 kg of milk/day with 3.8% fat and 3.7% protein (NRC, 2001). Rumen contents from each cow were transferred to a pre-warmed thermos for transport to the laboratory. Contents from both cows were combined in equal parts and strained through 4 layers of cheesecloth. Strained fluid was equally divided into 8 pre-warmed fermenters (1040 ± 20 ml strained fluid per fermenter). Twenty-five grams of pelleted experimental diet were added to fermenters immediately after inoculation.

Experimental Diets

A basal diet (Tables 2.1 and 2.2) was formulated to meet or exceed requirements for finishing feedlot cattle (NRC, 2000). All diet ingredients were mixed in a stationary mixer. The basal diet was dried at 60°C in a forced air oven for 48 h and ground in a Wiley No. 4 laboratory mill (Arthur H. Thomas Co., Philadelphia, PA) to pass a 2 mm screen. The ground diet was split into two equal portions and sodium sulfate was added to one portion to achieve a dietary sulfur content of 0.42%. Sulfur content of the unsupplemented portion was 0.21%. Each portion was divided a second time and bismuth subsalicylate (Sigma-Aldrich No. 480789, St Louis, MO) was added to the diet to

achieve concentrations of 0 (CON) or 0.5% (BSS) of diet DM. Diet preparation resulted in 4 treatments arranged in a 2 x 2 factorial design.

Each treatment diet was pelleted with a CL-5 California Pellet mill (California Pellet Mill Co., Crawfordsville, IN) to a final pellet dimension of 6 mm diameter x 12 mm long. Pelleted diets were placed in shallow trays and allowed to air dry for 96 h before storing in plastic containers.

Continuous Culture Operation

Eight dual flow continuous culture fermenters, described by Hannah et al. (1986) with a modified pH control and measuring system were used in two consecutive 10-d experimental periods. Treatments were randomly assigned within block (experimental period) to create a randomized complete block design and duplicated within blocks, resulting in 4 observations per treatment. Fermenters were provided with pelleted diet (described above) divided into eight equally spaced 90 min feedings using an automated feeding system. Amount of diet (as-fed) was adjusted on d 0, 4 and 7 for DM content to attain a feeding rate of 75 g of diet DM/d.

Artificial saliva (pH = 8.25) was prepared according to Weller and Pilgrim (1974) except for the replacement of $MgSO_4$ with $MgCl_2$ to a final composition (g/L) of Na_2HPO_4, 1.76; $NaHCO_3$, 5.0; KCl, 0.6; $MgCl_2$, 0.05; $KHCO_3$, 1.6; and urea, 0.4. Liquid flow rate of fermenters was set at 8.2% of fermenter volume (1040 ± 20 mL) per h by regulating artificial saliva input. Solids dilution rate was set at 4.1%/h by regulating liquid output through filters. Individual fermenter pH was measured every 5 minutes by an electric acquisition system (DasyLab v.5.04, DASYTEC USA, Bedford, NH).

Fermenter pH was maintained between 5.1 and 5.8 by automated addition of either 5N NaOH or 3N HCl. Anaerobic conditions were maintained with constant infusion of N_2 at a rate of 20 mL/min. Fermenter temperature was maintained at 39 ± 0.1°C by an electrical heater. Contents of fermenters were continuously agitated with a magnetic stir plate at 350 rpm.

Sample Collection

On the last 3 days of the experiment periods, fermenter solids and liquid effluents were collected in separate vessels and maintained at 1°C in a water bath to reduce microbial and enzymatic activities. Daily contents of liquid and solids effluent vessels were combined (within fermenter) and homogenized using a PT10/3S homogenizer (Kinematica GmbH, Bohemia, NY). A 500 mL subsample of combined solids and liquid effluent was composited daily by fermenter, resulting in a single combined effluent sample per fermenter representing 3 d of collection in each period. A portion of this sample was frozen and later thawed for total-N, NH_3-N and VFA analysis. Another portion of combined effluent was lyophilized and analyzed for DM, OM, NDF, ADF, ash, purines, amino acids and fatty acids. At the end of the 10-d experimental period, fermenter contents were filtered through 4 layers of cheesecloth and centrifuged at 1,000 x *g* to remove feed particles. Supernatant was subsequently centrifuged at 20,000 x *g* to isolate microbial cells, which were lyophilized and analyzed for DM, OM, total N and purines.

At 0700 hours on the same day as effluent collections, fermenter outflow and feed ports were sealed and nitrogen flow was adjusted to maintain 20 ± 1 mL/min using a

digital flow meter (Aalborg GFM 17, Orangeburgh, NY). After 20 min of stabilization, a 5 mL sample of fermenter headspace gas was taken through a septum using a gas tight syringe (Supelco, Bellefronte, PA) and transferred to a vaccutainer tube (Tyco, Mansfield, PA) containing 5 mL alkalized water (pH=9) for H_2S analysis.

Chemical Analysis

Dry matter and ash content of diets, lyophilized effluent and lyophilized microbial cells were determined by drying in an oven at 105°C for 24 h followed by combustion at 550°C for 6 h (AOAC 2005). Sequential detergent fiber analysis was conducted to determine NDF and ADF concentrations of diets and effluents using an ANKOM A200 fiber analyzer with F57 fiber bags (ANKOM Corp, Fairport, NY) and lignin content of the diet was measured gravimetrically after hydrolysis of acid detergent residue using 12 molar H_2SO_4 (Van Soest et al., 1991). Nitrogen content of the diets, liquid and freeze dried effluent, microbial cells and acid detergent residue were determined using the Kjeldahl method (AOAC, 1990). Purine concentration of effluent and microbial pellet was used to partition flows of effluent N into microbial and dietary N (Zinn and Owens, 1986). Ammonia concentration in the fermenters was determined on the supernatant of a centrifuged (5,000 x *g* for 15 min) sub-sample of liquid effluent by steam distillation (Bremner and Keeney, 1965) with magnesium oxide using a 2300 Kjeltec Analyzer Unit (Foss Tecator AB, Höganäs, Sweden).

Effluent VFA concentration was determined using gas-liquid chromatography. Fermenter effluent was clarified by centrifugation at 5,000 x *g* for 10 min. Two mL of supernatant was hydrolyzed using 0.5 mL of 25% m-phosphoric acid. The sample was

frozen at -20° C and thawed, followed by additional centrifugation at 5,000 x *g* for 10 min to remove hydrolyzed compounds. Clarified fluid was filtered through a polyethersulfone micro-pore filter with a 0.45 µm pore size. Analysis was performed using an HP 6890 GC (Hewlett-Packard, Palo Alto, CA) equipped an autosampler and a Supelco Carbopack glass column (2 m x 6.35 mm x 2 mm). Chromatographic conditions were: injection volume: 1.0 µL; injector temperature: 200°C; nitrogen (carrier gas): 24.6 mL/min; oven settings: initial temperature at 175°C, held for 21 min, ramped at 25°C/min to 200°C, held for 4 min, post run cool down held at 175°C for 4 min; flame ionization detector temperature was 230°C. Standard solutions with known concentrations of VFA were analyzed to calibrate chromatograph output and determine concentration of VFA in samples.

Hydrogen sulfide samples were analyzed according to Siegel (1965). Briefly, 0.5 mL of 4.7 m*M* 4-Amino-N,N-dimethylaniline sulfate and 0.5 mL of 98.9 m*M* ferric chloride was added to vaccutainers containing sample in alkalized water. Samples were allowed to develop and absorbance (665 nm) was determined using a Gilford Response spectrophotometer (Gilford Instrument Laboratories Inc., Oberlin, OH).

Amino acid content (excluding tryptophan) of lyophilized effluent was determined by the University of Missouri Agricultural Experiment Station according to AOAC (2006). Fatty acid analysis was performed by T. C. Jenkins at the University of Clemson. Sodium methoxide and methanolic HCl were used for direct trans-esterification of fatty acids to methyl esters (Jenkins, 2010) in lyophilized effluent samples. Fatty acid methyl esters were separated using an HP 5890A GC (Hewlett-Packard, Palo Alto, CA) equipped with an autosampler and an SP 2380 fused-silica capillary column (100 m x 0.25 mm i.d., 0.2-

μm film thickness). Chromatographic conditions were: helium (carrier and make-up gas): 20 cm/min; oven settings: initial temperature at 140°C, held for 4 min, ramped at 13°C/min to 160°C, held for 44 min, ramped at 4°C/min to 220°C, held for 20 min; flame ionization detector temperature was 250°C. Methyl ester peaks were identified based on comparison of their retention times to those of pure standards.

Statistical Analysis

All data processing and analysis were conducted using SAS software version 9.2 (SAS Institute Inc., Cary, NC, USA). Data from fermenters were analyzed as a randomized complete block design with a 2 x 2 factorial arrangement of treatments. Period served as block with all treatments equally represented within block. All parameters except fermentation pH (described below) were analyzed using the GLM procedure of SAS software. The model for each dependent variable was:

$$Y_{ij} = \mu + P_i + B_j + S_k + B_j \times S_k + \varepsilon_{ijk}$$

Where μ is the grand mean, P_i is the period (block), B_j is the BSS level, S_k is the sulfur level, $B_j \times S_k$ is the interaction term and ε_{ijk} is the random error. For all analysis, differences were considered significant at $P \leq 0.05$, with tendencies discussed at $0.1 \leq P > 0.05$. Differences among treatments were tested using LSMEANS with the PDIFF option in SAS. Results are reported as least squared means from 4 observations per treatment unless otherwise noted.

Fermentation pH recorded every 5 min obtained over three sampling days were summarized to determine simple mean, minimum and maximum pH on an hourly basis. These hourly statistics were analyzed as repeated measures using the MIXED procedure

of SAS software with a compound symmetry covariance structure. This covariance structure had the lowest AIC (Akaike's Information Criterion) value of several competing structures (Littel et al., 1998). Fixed effects in the model were dietary sulfur, BSS, and the interaction between the two factors. Random effect was fermenter nested within period.

Time (min) spent below pH 5.2, between pH 5.2 and 5.6, and above 5.6 were calculated using trapezoidal integration method. Minutes were calculated from the raw data set containing readings every 5 min. Comparisons between treatments were then conducted using the same methods as described above for all other data.

RESULTS AND DISCUSSION

Dry Matter, Organic Matter and Fiber Digestion

Inclusion of BSS in the diet decreased ($P < 0.001$) true DM and OM digestion in continuous culture compared with CON diets (Table 2.3). Neutral and acid detergent fiber digestion (Table 2.3) were not affected by treatment ($P > 0.05$). These results differ from Moreno (2012) who observed increased true OM digestion and fiber digestion with BSS in the diet. Values obtained by Moreno (2012) for NDF digestion were lower than ADF digestion and results from the present study display a similar inversion of NDF and ADF digestibilities. The same detergent fiber methodology was employed in both studies. These unrealistic relationships may be due to loss of fines after AD extraction. Raffrenato and Van Amburgh (2011) demonstrated a substantial increase in AD residue after addition of glass microfiber filters (1.5 μm pore size) to coarse Gooch crucibles (40-60

μm pore size). Alteration of the original Goering and Van Soest (1970) procedure increased AD residue measured in fecal samples by approximately 2 percentage points (Raffrenato and Van Amburgh, 2011). To test this concept, the basal diet and three randomly chosen effluent samples from the present study were subjected to sequential ND and AD extraction using a reflux apparatus and crucibles fitted with glass microfiber filters. These values were compared to those obtained using sequential AD and ND extraction outlined in materials and methods. Pore size of the ANKOM F57 bags was assumed to be 25 μm as per product literature. Acid detergent fiber concentration in diets was similar ($P > 0.05$) between methods. Acid detergent fiber concentration in effluents averaged 1.8 percentage points greater ($P < 0.05$) using the crucible and filter method. Greater loss of fines from effluent *vs.* feed samples may partially explain abnormalities in NDF *vs.* ADF digestion values in the present study.

While methodological considerations may be responsible for abhorrent NDF *vs.* ADF digestibilities, low overall fiber digestion values in this study are likely due to a decrease in fermentation pH. Hoover (1986) demonstrated that a reduction in pH had major effects on reducing fiber digestion while Yang et al. (2002) reported dramatic increases in fiber digestion as fermentation pH was artificially raised from 5.5 to 6.0. The exceptionally low fermentation pH of CON diets in the present study likely reduced fibrolytic activity in the fermenters. It is interesting to note higher fermentation pH associated with BSS inclusion did not result in concomitant enhancement of fiber digestion probably due to suppression of overall microbial activity associated with BSS inclusion.

VFA Production

Diets that contained BSS displayed a decrease ($P < 0.01$) in total VFA concentration (49.9 *vs.* 114.6 m*M*) and branched-chain VFA (0.03 *vs.* 0.32 m*M*) compared with CON diets (Table 2.4). Moreno (2012) recorded a more modest decrease of BSS inclusion on total VFA concentration but a 50% increase in branched-chain VFA with BSS inclusion. In the present study, molar proportions of acetate and propionate decreased ($P < 0.01$) with BSS addition, while butyric acid molar proportion increased ($P < 0.01$). These findings are in contrast with Moreno (2012), who observed increases in acetate and propionate and a decrease in butyrate molar proportions. Moreno (2012) also observed an increase ($P < 0.01$) in acetate to propionate ratio which is consistent with the current study. Discrepancies between Moreno (2012) and the current study are possibly due to different levels of BSS inclusion and its effects on OM and fiber digestion between experiments. Moreno (2012) had a greater concentration of distiller's grains, and probably higher levels of associated polyunsaturated fatty acids, which can have detrimental effects on fibrolytic bacteria, which are major producers of acetate (Zinn et al., 2000; Klopfenstein et al., 2008). Dietary sulfur level and interaction between sulfur and BSS in the current study had no influence ($P > 0.05$) on any VFA measurements. This is in contrast to Quinn et al. (2009) who demonstrated that dietary sulfur level can alter the response of VFA proportions to monensin in continuous culture; however results from Moreno (2012) indicated that BSS and monensin had very different effects on rumen microbes.

Nitrogen Metabolism

Addition of BSS at 0.5% of the diet had a marked impact on fermenter nitrogen metabolism, while dietary sulfur level had no effects ($P > 0.05$) (Table 2.5). Ammonia-N concentration in fermenter effluent increased ($P < 0.01$) from approximately 2.5 to 20.5 mg/dL with BSS inclusion in the diet. Ammonia-N concentrations of CON diets were very close to the minimum concentration of 2 mg NH_3-N/dL to support efficient microbial growth as recommended by Satter and Slyter (1974). Despite low NH_3-N concentrations, OM digestion and EMPS values were within the range of previous studies with similar levels of CP and CP degradability (Bach et al., 1999; Ariza et al., 2001; Lean et al., 2005). Other studies (Russell et al., 1983; Bach et al., 2008) reported no difference in microbial growth when NH_3-N concentrations were below 5 mg/dL. In the present study, low NH_3-N concentration observed with CON diets could have been due to a higher use of NH_3-N by starch-degrading microorganisms. Because NH_3-N is the only N source for cellulose-digesting bacteria, the low concentrations of NH_3-N associated with CON diets could have prevented greater growth of fibrolytic bacteria, resulting in reduced fiber digestion. In conclusion, it is possible that low observed NH_3-N concentrations limited microbial growth; however this effect was not manifested in a reduction of true OM digestion or efficiency of microbial protein synthesis.

Nitrogen flows (g/d) of NH_3-N and dietary N increased ($P < 0.01$) with BSS inclusion, while daily flows (g/d) of non-NH_3-N and microbial N decreased ($P < 0.01$). Effects of BSS inclusion on nitrogen flows are consistent with findings of Moreno (2012). Nitrogen flow data clearly indicate that BSS inclusion in the diet negatively affected nitrogen metabolism of ruminal microbes. Dietary CP degradation was lower (*P*

< 0.01) with BSS inclusion when compared to control diets (38 *vs.* 49% of CP intake). Moreno (2012) observed similar decreases in CP degradation; however values were greater compared with the present study which had relatively low pH. Calsamiglia et al. (2002) demonstrated a reduction in CP degradation when pH dropped below 6.0. In the present study pH was maintained between 5.1 and 5.8. Efficiency of microbial protein synthesis expressed as g of microbial N/kg of OM truly digested decreased ($P < 0.01$) with inclusion of BSS, indicating that ruminal microbes were not as efficient in incorporation of available nitrogen into biomass. Moreno (2012) observed similar decreases in EMPS when BSS was included at 1% of diet DM. Efficiency of microbial protein synthesis of CON diets were similar to previous studies in our lab. Overall, nitrogen metabolism data indicate that BSS inclusion decreased crude protein degradation and reduced microbial protein synthesis.

Fermentation pH

Mean, minimum and maximum fermentation pH increased ($P < 0.01$) with addition of BSS to the diet (Table 2.6). Mean fermentation pH was 5.73 for BSS diets, and 5.24 for CON diets. Dietary sulfur content had no effect on mean, minimum, or maximum fermentation pH ($P > 0.05$). Although fermentation pH was monitored and maintained between 5.1 and 5.8 by addition of 3 N HCl or 5 N NaOH, values that approached upper or lower limits were not observed and amount of acid and base pumped into fermenters during sampling days was very low (< 5 mL/d). The pH of initial inoculum from the rumen of donor cows at the start of period one and two was 5.3 and 5.2, respectively. Inclusion of BSS in the study by Moreno (2012) resulted in similar effects on

fermentation pH. Fermenter pH values in this experiment were slightly lower and are probably due to dietary differences.

Table 2.6 also displays length of time fermenters spent within specified ranges of pH < 5.2, 5.2 < pH > 5.6, and pH > 5.6. Inclusion of BSS greatly increased ($P < 0.01$) time spent above pH 5.6 while fermenters without BSS (CON) spent a greater ($P = 0.03$) amount of time below pH 5.2. No fermenters with BSS recorded a pH below 5.2 throughout the duration of the experiment. Fermentation pH of fermenters fed CON diets spent a greater ($P < 0.01$) amount of time in the pH range of 5.2 to 5.6. Dietary sulfur had no effect ($P > 0.05$) on time spent within specified ranges of fermentation pH. Specific pH ranges were chosen due to increasing risk of sub-acute ruminal acidosis as pH decreases below 5.6, with clinical signs of acute acidosis more common below pH 5.2 (Garrett et al., 1999). Fermentation pH could be considered low in the present study, however it is important to note that inconsistent feeding behavior increases risk of acidosis (Owens et al., 1998; Schwartzkopf-Genswein et al., 2004; Nagaraja and Lechtenberg, 2007) and feeding in the current study was consistent due to the use of an automated system.

Hydrogen Sulfide Release

BSS inclusion changed the color of fermenter contents from a light brown to a dark grey color. This is consistent with observations by Moreno (2012) and other published studies with human colonic digestions and indicates binding of sulfur compounds (Wachstein and Zak, 1946; Suarez et al., 1998). Hydrogen sulfide was not detected in the headspace samples for any treatment. Given fermenter color change with BSS treatments,

it is possible that H_2S was quantitatively bound, however lack of H_2S detection in fermenters fed CON diets is disconcerting, especially when dietary sulfur was 0.42% of diet DM. For this reason, a series of tests were performed. Reagents and equipment were found to be sufficient to detect H_2S release from a 50 μM solution of sodium sulfide, as described by Siegel (1965).

To further investigate lack of measurable H_2S release with all diets, subsamples of fermenter contents were collected through liquid effluent ports (10 mL/ fermenter) and acidified with 1 N HCl in a sealed serum bottle to ensure release of the hydrosulfide anion into gaseous H_2S, as this final reduction is favorable at lower pH (Bray and Till, 1975). Headspace analysis indicated no H_2S release. Finally, a 10 mL subsample was collected on d 10 from each fermenter and used to inoculate a batch culture containing 40 mL of artificial saliva (previously described) and 0.5 g of ground diet. Incubations proceeded for 24 h in a 125 mL serum bottle sealed with a butyl rubber stopper. Gas headspace measurements were taken (5 mL) and analyzed for H_2S. Hydrogen sulfide was not detected in any of the headspace samples. Results from tests suggest that H_2S concentration in fermenter headspace was either below detectable values (2 μ*M*) or compounds interfering with color formation were present. Siegel (1965) demonstrated that sodium bisulfate and sodium thiosulfate, present at 10 and 1 μ*M* concentrations (respectively), reduced absorbance of 665 nm wavelength up to 97% of sodium sulfide control. If either of these compounds were present in the sample, interference would result. However, the authors feel this is unlikely to have occurred, due to previous success in our lab with the H_2S measurement technique in fermentation vessel headspace (Moreno, 2012).

Severe reduction of H_2S accumulation in headspace samples may be due to other unknown compounds capable of binding sulfur and/or suppression of sulfate reducing bacteria. Molybdenum can bind sulfates (Bryden and Bray, 1974); however excess dietary molybdenum can be severely toxic and is not typically seen in high concentrations in feedlot diets (Kessler et al., 2012). Kung et al. (2000) demonstrated greater sulfide accumulation in batch cultures treated with monensin and attributed this observation to less competition for reducing equivalents by methanogens suppressed by monensin. This interaction is controversial (Quinn et al., 2009) and methanogen presence in continuous culture is reduced due to loss of protozoa (Mansfield et al., 1995). It is possible that low pH in CON diets resulted in severe reduction in counts of *Desulfovibrio desulfuricans*. Studies with sulfur reducing bacteria in non-ruminal cultures indicate that maximum yield is obtained at pH 7, although values lower than 6.8 were not tested (O'Flaherty et al., 1998). Fermentation pH in the present study was lower than the Moreno (2012) study and could partially explain lack of H_2S accumulation in CON diets if sulfate reducing bacteria were inhibited by low pH, although data is lacking to determine minimum tolerable pH for sulfate reducing bacteria in ruminants.

Ultimately, lack of measureable H_2S release in the present study cannot be clearly explained with the data presented. Existing literature indicate that poor H_2S detection could be due to a combination of inhibition of sulfate reducers (possibly pH dependent), presence of other unknown molecules binding sulfur compounds and interference in assay colorimetric change by unknown molecules present in fermenter headspace samples.

Amino Acid Flows

Daily amino acid intake was calculated by multiplying amino acid content of the diet by daily DM fed (Table 2.7). Flows were determined by multiplying measured outflow (g/d) by AA content of the effluent for each fermenter (Table 2.8). Bismuth subsalicylate reduced ($P < 0.01$) net flow (g/d) for all measured amino acids except Cys, which increased and Pro, which remained the same as CON. High dietary sulfur decreased ($P < 0.05$) daily flow of Pro and Cys compared to low sulfur diets. The decrease in flows of Cys and the numerical decrease in Met flow with high sulfur treatments is a perplexing result, considering other metabolic fates of sulfur (H_2S). In the present study, amino acid profile was not determined on the microbial pellet due to insufficient sample amount. Due to large amount of variation in microbial AA flow dependent on diet (Clark et al., 1992) it is difficult to attribute relative changes of specific AA without knowledge of microbial AA profile for each treatment. Relative to dietary intakes of amino acids, all amino acids increased because of the contribution of microbial amino acid synthesis. Reduction in AA flows associated with BSS inclusion can be attributed to the overall negative effects on fermentation that were observed, culminating in a reduction in microbial protein synthesis. Because of the large impact of BSS inclusion on rumen nitrogen metabolism, it is likely that individual species of bacteria were altered due to relative levels of peptides and NH_3-N. Cotta and Hespell (1986) demonstrated that high levels of amino acids or peptides in ruminal fluid distinctly reduced proteolytic activity of *Butyrivibrio firbrisolvens*. Ruminal microbes have been shown to have varying efficiencies in utilization of AA and peptides depending on availability of energy and nitrogen (Cotta and Russell, 1982; Bach and Stern, 1999). A lower efficiency of AA

utilization can be detrimental to achieving high animal production because microbial protein synthesis is not maximized, resulting in an increased need for RUP or expensive protected AA supplements (Chalupa, 1975; Schwab, 1996).

Effluent Fatty Acid Flows from Fermenters

Fatty acid content of effluents is reported in Table 2.9. Daily intakes and outflows are presented in Table 2.10. Inclusion of BSS increased ($P < 0.05$) proportion (g/100 g total FA) of $C_{18:2}$ and $C_{18:3}$ in effluent samples. However, daily FA flows (g/d) increased ($P < 0.05$) probably due to reduced OM digestion resulting in reduced biohydrogenation. Daily flows of total $C_{18:1}$ and the *trans* isomer decreased ($P < 0.05$) with BSS inclusion and dietary sulfur level. Stearic acid flows were greatest in CON diets with high dietary sulfur, while *cis*-$C_{18:1}$ was highest in CON diets with low dietary sulfur. Influence of sulfur level on fatty acid metabolism is not well understood. Many studies focus on sulfur-associated polioencephalomalacia risks, although some authors reported negative effects on rumen fermentation when dietary sulfur was greater than 0.2% of diet DM (Johnson et al., 1971; Zinn et al., 1997; Spears, 2003).

Overall fatty acid flows indicate that BSS decreased biohydrogenation in continuous culture, likely through changes in overall microbial activity. Interestingly, *cis*-$C_{18:1}$ was greater ($P < 0.05$) with CON diets, although this was influenced by dietary sulfur level. Biohydrogenation of oleic acid can proceed directly to stearic acid, however *cis/trans* isomerization is common by some rumen microorganisms (Jenkins et al., 2008). The degree to which isomerization occurs is likely due to environmental conditions. In continuous culture AbuGhazaleh et al. (2005) demonstrated lower isomerization to *trans*

monoenes when culture pH was low. This observation is consistent with those found in the current study, as *trans*-$C_{18:1}$ was greater in BSS treatments where pH was elevated. Cellulolytic bacteria such as *Butyrivibrio fibrisolvens* are the main bacteria responsible for biohydrogenation (Harfoot and Hazlewood, 1997). In pure culture, Russell and Dombrowski (1980) demonstrated 75% of maximum yield for *Butyrvibrio fibrisolvens* at pH 5.75, however when culture pH was reduced to 5.5, the organism was washed out, possibility due to inability of the organism to attach to substrate at low pH conditions (Martin et al., 2002). In the present study, reduced isomerization of C_{18-1} in CON diets could be related to *Butyrvibrio fibrosolvens* suppression at low pH. Fatty acid data from fermenters indicate that BSS had strong effects on fatty acid biohydrogenation, likely mediated through effects on fermentation pH. Effects of dietary sulfur level on biohydrogenation are not well understood and further investigation may be useful.

CONCLUSIONS

Inclusion of BSS at 0.5% of diet DM had strong effects on fermentation by ruminal microbes in continuous culture. Digestion, VFA production, N metabolism and fermentation pH data indicates that BSS suppressed overall fermentation. Amount of dietary sulfur (0.21 and 0.42 % of diet DM) had no effect on major indicators of fermentation however moderate effects were detected on fatty acid metabolism. Results from the current study demonstrate that dose-titration studies in vitro and in vivo are needed to determine whether BSS addition to a ruminant diet can elicit positive responses to fermentation while binding hydrogen sulfide.

Table 2.1. Ingredient and chemical composition of basal experimental diet

Item	Composition[1]
Feed composition	
Dry rolled corn	39.0
Earlage	32.1
Wet distiller's grains	21.2
Corn silage	3.2
Supplement[2]	2.6
Urea premix[3]	2.0
Chemical composition	
Crude protein	15.9
NDF	20.6
ADF	8.6
Lignin	1.0
Ash	5.3
Starch	45.8
Crude fat	5.1

[1]Composition as % of 100°C DM unless otherwise noted.

[2]Supplement contained (DM basis): monensin, 0.8 g/kg; Ca, 25%; P, 0.1%; NaCl, 7%; K, 3%; Co, 10 mg/kg; Cu, 350 mg/kg; Se, 9 mg/kg; Zn, 1750 mg/kg; vitamin A 15,000 IU/kg; vitamin D_3, 29,000 IU/kg; vitamin E, 165 IU/kg; Thiamine, 500 mg/kg.

[3]Premix contained (DM basis): soybean meal, 714 g/kg; urea 286 g/kg.

Table 2.2. Fatty acid and amino acid composition of the basal diet

Item	Composition
Total fatty acids, g/100 g of DM	4.9
Individual fatty acids, g /100 g of total FA	
$C_{16:0}$	14.5
$C_{18:0}$	1.8
Total $C_{18:1}$	23.1
trans-$C_{18:1}$	23.1
cis-$C_{18:1}$	0.0
$C_{18:2}$	50.0
$C_{18:2}$ conjugated	0.0
$C_{18:3}$	1.5
Other	8.1
Total amino acids, mg/g of DM	125.7
Essential amino acids, mg/g of DM	
Threonine	4.9
Valine	6.7
Methionine	2.7
Isoleucine	5.0
Leucine	14.8
Phenylalanine	6.6
Histidine	3.1
Lysine	4.3
Arginine	5.7
Nonessential amino acids, mg/g of DM	
Aspartic acid	9.4
Serine	5.8
Glutamic acid	22.1
Proline	10.0
Glycine	5.4
Alanine	9.8
Cysteine	2.3
Tyrosine	4.8
Other amino acids, mg/g of DM[1]	2.3

[1]Composition, g/g of other amino acids: taurine, 0.33; hydroxyproline, 0.14; hydroxylysine, 0.43; ornithine, 0.10.

Table 2.3. Effect of bismuth subsalicylate and dietary sulfur level on DM, OM and fiber digestion in continuous culture

	Treatment[1]							
	Low sulfur		High sulfur			*P*- value		
Item, g/100 g	CON	BSS	CON	BSS	SEM[2]	BSS	Sulfur	B x S
DM, apparent	47.1	39.2	50.3	39.3	1.4	<0.01	0.31	0.38
DM, true[4]	64.1	46.9	67.9	47.6	2.5	<0.01	0.13	0.33
OM, apparent	37.5	26.9	41.4	28.2	1.8	<0.01	0.13	0.46
OM, true[4]	52.8	34.1	57.2	35.9	2.7	<0.01	0.13	0.33
NDF	19.3	27.5	24.4	30.0	4.5	0.15	0.41	0.77
ADF	32.1	35.0	36.7	38.2	2.9	0.47	0.21	0.81

[1]Treatments were arranged in a 2 x 2 factorial design, with dietary sulfur at 0.12(Low sulfur) or 0.42(High sulfur) % of diet DM, and bismuth subsalicylate at 0(CON) and 0.5%(BSS) of diet DM. High sulfur level was achieved by addition of sodium sulfate to the basal diet.

[2]Standard error of the mean, n = 4 replicates for all treatments.

Table 2.4. Effect of bismuth subsalicylate and dietary sulfur level on VFA concentration in continuous culture

	Treatment[1]							
	Low sulfur		High sulfur			*P*- value		
Item, g/100 g	**CON**	**BSS**	**CON**	**BSS**	**SEM[2]**	**BSS**	**Sulfur**	**B x S**
Total VFA (m*M*)	107.0	49.7	122.3	50.1	9.7	<0.01	0.39	0.42
Individual VFA (mol/100mol)								
Acetate	34.5	27.5	34.5	29.8	1.2	0.01	0.56	0.57
Propionate	36.1	13.8	39.0	20.7	3.4	<0.01	0.27	0.65
Butyrate	14.4	33.3	13.7	25.9	2.5	<0.01	0.19	0.27
Isobutyrate	0.12	0.03	0.15	0.10	0.02	0.15	0.27	0.61
Isovalerate	0.15	0.0	0.11	0.0	0.03	<0.01	0.62	0.62
Branched-chain VFA (m*M*)	0.28	0.01	0.36	0.04	0.05	<0.01	0.46	0.78
A:P Ratio	0.99	2.16	0.98	1.47	0.15	<0.01	0.12	0.12

[1]Treatments were arranged in a 2 x 2 factorial design, with dietary sulfur at 0.12(Low sulfur) or 0.42(High sulfur) % of diet DM, and bismuth subsalicylate at 0(CON) and 0.5%(BSS) of diet DM. High sulfur level was achieved by addition of sodium sulfate to the basal diet.

[2]Standard error of the mean, n = 4 replicates for all treatments.

Table 2.5. Effect of bismuth subsalicylate (BSS) and dietary sulfur level on nitrogen metabolism in continuous culture

	Treatment[1]							
	Low sulfur		High sulfur			*P*- value		
Item	**CON**	**BSS**	**CON**	**BSS**	**SEM[2]**	**BSS**	**Sulfur**	**B x S**
NH_3-N (mg/dL)	2.7	20.8	2.4	19.9	2.3	<0.01	0.37	0.67
N flow (g/d)								
NH_3-N	0.05	0.40	0.05	0.38	0.04	<0.01	0.36	0.69
Non NH_3-N	2.10	1.72	2.06	1.67	0.05	<0.01	0.21	0.88
Microbial-N	1.10	0.50	1.09	0.52	0.08	<0.01	0.78	0.67
Dietary-N	1.00	1.22	0.97	1.15	0.03	0.02	0.32	0.69
CP degradation (%)	47.6	36.2	49.4	40.0	2.4	<0.01	0.28	0.69
EMPS[4]	29.3	20.6	26.8	20.7	0.8	<0.01	0.14	0.13

[1]Treatments were arranged in a 2 x 2 factorial design, with dietary sulfur at 0.12(Low sulfur) or 0.42(High sulfur) % of diet DM, and bismuth subsalicylate at 0(CON) and 0.5%(BSS) of diet DM. High sulfur level was achieved by addition of sodium sulfate to the basal diet.

[2]Standard error of the mean, n = 4 for all treatments.

[3]EMPS: Efficiency of microbial protein synthesis (g of microbial N/kg of OM truly digested).

Table 2.6. Effect of bismuth subsalicylate (BSS) and dietary sulfur level on fermentation pH in continuous culture

	Treatment[1]							
	Low sulfur		High sulfur			*P*- value		
Item	CON	BSS	CON	BSS	SEM[2]	BSS	Sulfur	B x S
Mean pH[3]	5.23	5.75	5.25	5.72	0.02	<0.01	0.77	0.26
Minimum pH[3]	5.17	5.70	5.19	5.68	0.03	<0.01	0.95	0.50
Maximum pH[3]	5.30	5.80	5.32	5.77	0.02	<0.01	0.81	0.20
Time below pH 5.2[4]	1.9	4276.5	0.9	3814.3	485.2	<0.01	0.31	0.37
Time between pH 5.2 and 5.6[4]	1102.6	0.0	1032.4	0.0	772.2	0.03	0.93	0.93
Time above pH 5.6[4]	3210.5	38.2	3281.6	500.7	913.4	<0.01	0.58	0.68

[1]Treatments were arranged in a 2 x 2 factorial design, with dietary sulfur at 0.12(Low sulfur) or 0.42(High sulfur) % of diet DM, and bismuth subsalicylate at 0(CON) and 0.5%(BSS) of diet DM. High sulfur level was achieved by addition of sodium sulfate to the basal diet.

[2]Standard error of the mean.

[3]Analyzed as repeated measures with 1 observation/fermenter/hour during 3 consecutive sampling days.

[4]Total minutes during 3 consecutive days, n = 4 replicates per treatment.

Table 2.7. Daily dietary amino acid intake[1]

	Treatment[2]			
	Low sulfur		High sulfur	
Amino acids	**CON**	**BSS**	**CON**	**BSS**
Total, g/d	9.44	9.42	9.41	9.42
Essential, g/d	4.03	4.02	4.02	4.02
Nonessential, g/d	5.41	5.40	5.39	5.40
Essential, mg/d				
Threonine	367.3	366.4	366.2	366.4
Valine	500.9	499.6	499.3	499.7
Methionine	200.3	199.8	199.7	199.9
Isoleucine	375.6	374.7	374.5	374.8
Leucine	1110.2	1107.4	1106.9	1107.6
Phenylalanine	492.5	491.3	491.0	491.4
Histidine	233.7	233.1	233.0	233.2
Lysine	325.6	324.7	324.6	324.8
Arginine	425.7	424.6	424.4	424.7
Nonessential, mg/d				
Aspartic Acid	709.5	707.7	707.4	707.9
Serine	434.1	433.0	432.8	433.1
Glutamic Acid	1661.2	1657.0	1656.2	1657.3
Proline	751.3	749.4	749.0	749.5
Glycine	409.0	408.0	407.8	408.1
Alanine	734.6	732.7	732.4	732.9
Cysteine	175.3	174.9	174.8	174.9
Tyrosine	358.9	358.0	357.9	358.1
Other nonessential, mg/d	175.3	174.9	174.8	174.9

[1]Daily intakes calculated as diet DM intake x amino acid concentration of diet.
[2]Treatments were arranged in a 2 x 2 factorial design, with dietary sulfur at 0.12(Low sulfur) or 0.42(High sulfur) % of diet DM, and bismuth subsalicylate at 0(CON) and 0.5%(BSS) of diet DM. High sulfur level was achieved by addition of sodium sulfate to the basal diet.

Table 2.8. Effect of bismuth subsalicylate (BSS) and dietary sulfur level on amino acid flow from fermenters[1]

	Treatment[2]							
	Low sulfur		High sulfur			*P*- value		
Amino acid	CON	BSS	CON	BSS	SEM[3]	BSS	Sulfur	B x S
Essential flow, mg/d								
Threonine	528.2	396.3	498.8	394.0	11.5	<0.01	0.24	0.34
Valine	667.3	551.4	634.8	538.0	11.2	<0.01	0.18	0.66
Methionine	262.4	197.3	246.5	197.0	7.2	<0.01	0.42	0.45
Isoleucine	554.2	435.1	528.9	424.2	9.8	<0.01	0.20	0.70
Leucine	1334.8	1152.2	1257.3	1137.9	26.0	<0.01	0.18	0.39
Phenylalanine	638.2	514.4	598.2	509.4	11.5	<0.01	0.15	0.28
Histidine	253.9	237.9	242.9	231.2	4.8	0.03	0.19	0.85
Lysine	513.8	431.4	537.2	415.3	20.6	<0.01	0.83	0.36
Arginine	510.8	433.3	504.0	422.6	12.6	<0.01	0.62	0.77
Nonessential flow, mg/d								
Aspartic acid	1077.0	792.7	1029.5	785.9	20.4	<0.01	0.31	0.50
Serine	540.3	423.9	510.1	428.6	14.2	<0.01	0.47	0.32
Glutamic acid	1919.7	1690.9	1797.2	1667.2	32.2	<0.01	0.10	0.29
Proline	743.1	726.7	685.6	704.5	14.4	0.96	0.04	0.39
Glycine	541.9	427.8	510.7	424.5	11.0	<0.01	0.24	0.36
Alanine	931.4	759.6	883.6	767.4	14.5	<0.01	0.36	0.20
Cysteine	168.1	171.5	153.4	166.2	3.0	0.02	0.01	0.22
Tyrosine	514.6	403.7	487.5	399.1	9.9	<0.01	0.22	0.43

[1]Daily outflows calculated as effluent DM outflow x amino acid concentration of effluent.

[2]Treatments were arranged in a 2 x 2 factorial design, with dietary sulfur at 0.12(Low sulfur) or 0.42(High sulfur) % of diet DM, and bismuth subsalicylate at 0(CON) and 0.5%(BSS) of diet DM. High sulfur level was achieved by addition of sodium sulfate to the basal diet.

[3]Standard error of the mean, n = 4 replicates for all treatments.

Table 2.9. Effect of bismuth subsalicylate (BSS) and dietary sulfur level on fatty acid profile of effluent from fermenters

	Treatment[1]				
	Low sulfur		High sulfur		
Fatty acids	**CON**	**BSS**	**CON**	**BSS**	**SEM[2]**
Total, % of DM	5.23	5.75	5.25	5.72	0.02
Individual, % of total fatty acids					
$C_{16:0}$	15.3	15.7	14.0	14.4	0.8
$C_{18:0}$[b]	6.6[c]	1.9[c]	13.4[d]	1.9[c]	1.3
Total $C_{18:1}$[b]	52.2[c]	24.3[e]	47.9[d]	25.2[e]	1.0
trans-$C_{18:1}$[b]	19.3[c]	24.1[d]	17.8[c]	24.9[d]	0.4
cis-$C_{18:1}$[a]	32.9	0.2	30.1	0.3	0.8
$C_{18:2}$[a]	19.8	53.0	19.0	54.1	1.0
$C_{18:2}$ Conjugated	0.02	0.02	0.01	0.01	0.01
$C_{18:3}$[a]	0.54	1.95	0.54	1.66	0.2
Other[a]	4.2	2.0	3.9	1.6	0.2

[a]Main effect of bismuth subsalicylate ($P < 0.05$).

[b]Interaction of bismuth subsalicylate and dietary sulfur ($P < 0.05$).

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

[1]Treatments were arranged in a 2 x 2 factorial design, with dietary sulfur at 0.12 or 0.42% of diet DM, and bismuth subsalicylate at 0(CON) or 0.5%(BSS) of diet DM.

[2]Standard error of the mean, n = 4 replicates per treatment.

Table 2.10. Effect of bismuth subsalicylate (BSS) and dietary sulfur level on fatty acid flows from fermenters

	Treatment[1]				
	Low sulfur		High sulfur		
Item	**CON**	**BSS**	**CON**	**BSS**	**SEM[2]**
Total fatty acid intake, g/d[3]	3.71	3.70	3.70	3.70	-
Individual fatty acid intake, g/d					
$C_{16:0}$	0.54	0.54	0.54	0.54	-
$C_{18:0}$	0.07	0.07	0.07	0.07	-
Total $C_{18:1}$	0.86	0.85	0.85	0.86	
$C_{18:2}$	1.86	1.85	1.85	1.85	-
$C_{18:2}$ Conjugated	-	-	-	-	-
$C_{18:3}$	0.06	0.06	0.06	0.06	-
Other	0.30	0.30	0.30	0.30	-
Total fatty acid flow, g/d[a4]	3.12	3.90	2.90	3.42	0.17
Individual fatty acid flow, g/d					
$C_{16:0}$	0.48	0.62	0.40	0.50	0.04
$C_{18:0}$[c]	0.21[d]	0.07[d]	0.40[e]	0.06[d]	0.06
Total $C_{18:1}$[a,b]	1.63	0.94	1.39	0.86	0.06
trans-$C_{18:1}$[a,b]	0.60	0.94	0.52	0.85	0.03
cis-$C_{18:1}$[c]	1.03[d]	0.01[f]	0.87[e]	0.01[f]	0.04
$C_{18:2}$[a]	0.62	2.05	0.55	1.85	0.05
$C_{18:2}$ Conjugated	-	-	-	-	-
$C_{18:3}$[a]	0.02	0.08	0.02	0.06	0.01
Other[a,b]	0.13	0.08	0.11	0.05	0.01

[a]Main effect of bismuth subsalicylate ($P < 0.05$).

[b]Main effect of dietary sulfur ($P < 0.05$).

[c]Interaction of bismuth subsalicylate and dietary sulfur ($P < 0.05$).

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

[1]Treatments were arranged in a 2 x 2 factorial design, with dietary sulfur at 0.12 or 0.42% of diet DM, and bismuth subsalicylate at 0(CON) or 0.5%(BSS) of diet DM.

[2]Standard error of the mean, n = 4 replicates per treatment.

[3]Daily intakes calculated as diet DM intake x fatty acid concentration of the diet.

[4]Daily outflows calculated as effluent DM outflow x fatty acid concentration of effluent.

EXPERIMENT 2: Effects of beta-extract of *Humulus lupulus* (hops) on fermentation by ruminal microbes in continuous culture

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Abstract. Ionophores are commonly used in ruminant production to selectively inhibit ruminal microorganisms and promote a more efficient fermentation. Beta-acids in hops (*Humulus lupulus*) have been shown to exhibit selective bacteriostatic properties toward Gram-positive and hyper-ammonia producing bacteria in pure and co-culture. Previous work with in vitro batch cultures containing whole hop and hop extracts demonstrated promising results in altering fermentation. This study used eight dual-flow continuous culture fermenters in two consecutive 10-d periods consisting of 7 d of adaptation followed by 3 d of sampling. A basal diet containing 44 % corn silage, 14% alfalfa hay, 13 % ground corn, 11 % protein mix, 10 % corn gluten feed, 5 % cottonseed, and 3 % liquid vitamin and mineral supplement on a DM basis was provided to the fermenters at a rate of 75 g of DM/L of fermenter volume/d. Hop beta-extract (BE) was added daily to the artificial saliva to supply 0 (CON), 600 (LOW), 1200 (MED), or 1800 (HIGH) mg of β-acids/kg of diet DM/day. Effluents from sampling days were composited by fermenter within period, resulting in 4 reps/treatment. Beta extract inclusion had no effects on DM, OM, or fiber digestion ($P > 0.05$). VFA production and

N metabolism were not affected by BE inclusion ($P > 0.05$). Mean and maximum fermentation pH tended ($P = 0.09$) to increase linearly with increasing levels of BE inclusion. Time spent above pH 6.2 tended to increase linearly with greater BE inclusion ($P = 0.08$), while time spent between pH 5.8 and 6.2 tended to decrease linearly ($P = 0.07$). Changes in pH were less than biologically relevant levels. Increasing levels of BE had very limited effects on fermentation measurements by ruminal microbes using continuous culture fermenters.

Keywords: continuous culture, hops, beta-acids, rumen

INTRODUCTION

Hops (*Humulus lupulus*) are commonly used as a flavor, aroma, and preservation agent in the brewing of beer. Naturally occurring hop compounds, namely humulones (α-acids) and lupulones (β-acids), exhibit bacteriostatic properties in brewing applications (Hastings and Walker, 1929; Haas and Barsoumian, 1994). The mode of action of hop acids is likely due to their protonophoric characteristics in lipid bilayers (Sakamoto and Konings, 2003; Gerhäuser, 2005).

Spent hops from the brewing process are often fed to cattle with brewers grains. Effects of spent hops on rumen fermentation have been examined by Krishna et al. (1986). During the brewing process, α and β-acids are solubilized and removed from the residue, reducing possible effects of spent hops on rumen bacterial species. Some in vitro fermentation research with rumen microbes have used whole or ground hops, but confounding factors of additional fermentable matter and other antimicrobial compounds such as condensed tannins limit the ability to properly describe effects of hop acids on

fermentation with rumen microbes (Narvaez et al., 2011). Recently, supercritical CO_2 extraction of α-and β-acids has allowed for more targeted research on effects of hop extracts. Flythe (2009) demonstrated suppression of ruminal hyper ammonia-producing bacteria in pure culture when hop beta-extract (BE) was included in fermentation medium, while others have shown a decrease in methane emission with BE inclusion with mixed ruminal fermentations (Narvaez et al., 2012, 2013).

In vivo studies have been inconsistent in regard to effects of hops and BE on ruminal fermentation. Wang et al. (2010) observed no effects on rumen fermentation, carcass characteristics or shedding of *Escherichia coli* when hop pellets were fed to feedlot cattle. Kaneda et al. (1990) reported that aerobic stability of hop acids is often an issue when supplied in pelleted form. Results of previous research suggest that hop extracts should be supplied consistently and without aerobic exposure to elicit meaningful alterations to rumen fermentation. The objective of this study was to evaluate the effects of increasing amounts of BE on in vitro fermentation with ruminal microbes in continuous culture.

MATERIALS AND METHODS

Animals and Collection of Rumen Fluid

The University of Minnesota Institutional Animal Care and Use Committee approved all animal use in this study. Two ruminally cannulated lactating dairy cows served as rumen fluid donors. Composition of the diet fed to donor cows was formulated to be the same as the experimental diet(Table 3.1), and contained approximately 44 % corn silage, 14% alfalfa hay, 13 % ground corn, 11 % protein mix, 10 % corn gluten feed, 5 %

cottonseed, and 3 % liquid vitamin and mineral supplement (DM basis). Rumen contents from each cow were collected into a pre-warmed thermos and transported to the laboratory. Contents from both cows were combined in equal parts and strained through 4 layers of cheesecloth. Strained fluid was equally divided into 8 pre-warmed fermenters (1040 ± 20 mL per fermenter). Twenty-five grams of pelleted diet were added to fermenters immediately after inoculation.

Diet and Treatments

A basal diet was formulated to meet or exceed requirements of a Holstein cow producing 40 kg of milk/day, with 3.8% fat and 3.7% protein (NRC, 2001). After mixing, the diet was dried at 60°C in a forced air oven for 48 h and ground in a Wiley No. 4 laboratory mill (Arthur H. Thomas Co., Philadelphia, PA) to pass a 2 mm screen. The ground diet was pelleted with a CL-5 California Pellet mill (California Pellet Mill Co., Crawfordsville, IN) to a final pellet dimension of 6 mm diameter x 12 mm long. Pelleted diet was placed in shallow trays to air dry for 96 h before storing in plastic containers. Dry matter content was determined periodically on pelleted diets during the experimental periods.

Hop beta-extract was provided as a 45% (± 1%) β-acid solution with propylene glycol (PG), as determined by HPLC by the supplier (S.S. Steiner, Inc., New York, NY). Beta-extract was added daily to the artificial saliva to supply 0 (CON), 600 (LOW), 1200 (MED), and 1800 (HIGH) mg of β-acids/kg of diet DM/day. Additional PG equal to the amount introduced via the BE solution for the HIGH treatment was added to the CON, LOW and MED treatments to supply 2200 mg PG/kg of diet DM/day for all treatments.

Artificial saliva containing the treatments was mixed fresh daily. Beta-extract stock solution and final artificial saliva solutions were stored in the dark throughout the experimental periods. Beta extract dose was chosen based off recommendations from previous in vitro experimentation (Narvaez et al., 2011).

Continuous Culture Operation

Eight dual flow continuous culture fermenters, described by Hannah et al. (1986) with a modified pH control and measuring system were used in two consecutive 10-d experimental periods. Treatments were randomly assigned within block (experimental period) to create a randomized complete block design. Treatments were duplicated within blocks, resulting in 4 observations per treatment. Fermenters were provided with 75 g of DM/L of fermenter volume/d of pelleted diet divided into eight equally spaced 90 min feedings using an automated feeding system.

Artificial saliva (pH = 8.25) was prepared according to Weller and Pilgrim (1974) to a final composition (g/L) of Na_2HPO_4, 1.76; $NaHCO_3$, 5.0; KCl, 0.6; $MgSO_4$, 0.05; $KHCO_3$, 1.6; and urea, 0.4. Liquid flow rate of fermenters was set at 10% of fermenter volume (1040 ± 20 mL) per h by regulating artificial saliva input. Solids dilution rate was set at 5.5%/h by regulating liquid output through filters. Individual fermenter pH was measured every 5 minutes by an electric acquisition system (DasyLab v.5.04, DASYTEC USA, Bedford, NH). Fermenter pH was maintained between 5.6 and 6.4 by automated addition of either 5N NaOH or 3N HCl. Anaerobic conditions were maintained with constant infusion of N_2 at a rate of 20 mL/min. Fermenter temperature was maintained at

39 ± 0.1°C by an electrical heater. Contents of fermenters were agitated with a magnetic stir plate at 350 rpm.

Sample Collection

There was a 7 d adaptation period followed by 3 d of sample collection. Fermenter solids and liquid effluents were collected in separate vessels and maintained at 1°C in a water bath to reduce microbial and enzymatic activities on the last 3 d. On sampling days contents of liquid and solids effluent vessels were combined (within fermenter) and homogenized using a PT10/3S homogenizer (Kinematica GmbH, Bohemia, NY). A 500 mL subsample of combined solids and liquid effluent was composited daily by fermenter, resulting in a single combined effluent sample per fermenter representing 3 d of collection in each period. A portion of this sample was frozen and later thawed for total-N, NH_3-N and VFA analysis. Another portion of effluent was lyophilized and analyzed for DM, OM, NDF, ADF, ash and purines. At the end of the 10-d experimental period, fermenter contents were filtered through 4 layers of cheesecloth and centrifuged at 1,000 x *g* to remove feed particles. Clarified contents were centrifuged at 20,000 x *g* to isolate microbial cells, which were lyophilized and analyzed for DM, OM, total N and purines.

Chemical Analysis

Dry matter of diets, lyophilized effluent and lyophilized microbial cells were determined by drying in an oven at 105°C for 24 h. Amount of diet (as-fed) was adjusted on d 0, 4, and 7 for DM content to attain a feeding rate of 75 g of diet DM/L of fermenter volume/d.

Dry matter and ash content of diets, lyophilized effluent and lyophilized microbial cells were determined by drying in an oven at 105°C for 24 h followed by combustion at 550°C for 6 h (AOAC 2005). Sequential detergent fiber analysis (Van Soest et al., 1991) was conducted to determine NDF and ADF concentrations of diets and effluents using an ANKOM A200 fiber analyzer with F58 fiber bags (ANKOM Corp, Fairport, NY). Nitrogen content of the diets, liquid effluent, and microbial cells was determined using the Kjeldahl method (AOAC, 1990). Purine concentration of the effluent and microbial pellet was used to partition flows of effluent N into microbial and dietary N (Zinn and Owens, 1986). Ammonia concentration in the fermenters was determined on the supernatant of a centrifuged (5,000 x *g* for 15 min) sub-sample of liquid effluent by steam distillation with magnesium oxide using a 2300 Kjeltec Analyzer Unit (Foss Tecator AB, Höganäs, Sweden).

Effluent VFA concentration was determined using gas-liquid chromatography. Fermenter effluent was clarified by centrifugation at 5,000 x *g* for 10 min. Supernatant was hydrolyzed using a solution of 25% m-phosphoric, frozen at -20° C and thawed, followed by additional centrifugation at 5,000 x *g* for 10 min to remove hydrolyzed proteins. Clarified fluid was filtered through a polyethersulfone micro-pore filter with a 0.45 µm pore size. Analysis was performed using an HP 6890 GC (Hewlett-Packard, Palo Alto, CA) equipped an autosampler and a Supelco Carbopack glass column (2 m x 6.35 mm x 2 mm). Chromatographic conditions were: injection volume: 1.0 µL; injector temperature: 200°C; nitrogen (carrier gas): 24.6 mL/min; oven settings: initial temperature at 175°C, held for 21 min, ramped at 25°C/min to 200°C, held for 4 min, post run cool down held at 175°C for 4 min; flame ionization detector temperature was

230°C. Standard solutions with known concentrations of VFA were analyzed to calibrate chromatograph output and determine concentration of VFA in samples.

Statistical Analysis

All data processing and analysis were conducted using SAS software version 9.2 (SAS Institute Inc., Cary, NC, USA). Data from fermenters were analyzed as a randomized complete block design. Period served as block with all treatments equally represented within block. All parameters except fermentation pH (described below) were analyzed using the GLM procedure of SAS software. For all analysis, differences were considered significant at $P \leq 0.05$, with tendencies discussed at $0.1 \leq P > 0.05$. Differences among treatments were tested using LSMEANS with the PDIFF option in SAS. Results are reported as least squared means from 4 observations per treatment unless otherwise noted. Differences among treatments were reported as polynomial contrasts to determine linear and/or quadratic responses to BE concentration. Contrast of control *vs.* BE treatment were tested using LSMEANS with the PDIFF option, however results not shown due to insignificant results.

Fermentation pH recorded every 5 min obtained over three sampling days were summarized to determine simple mean, minimum and maximum pH on an hourly basis. These hourly statistics were analyzed as repeated measures using the MIXED procedure of SAS software with a compound symmetry covariance structure. This covariance structure had the lowest AIC (Akaike's Information Criterion) value of several competing structures (Littel et al., 1998). Beta-extract level was treated as a fixed effect in the model, and fermenter nested within period was a random effect.

Time (min) spent below pH 5.2, between pH 5.2 and 5.6, and above 5.6 were calculated using trapezoidal integration method. Minutes were calculated from the raw data set containing readings every 5 min. Comparisons between treatments were then conducted using the same methods as described above for all other data.

RESULTS AND DISCUSSION

Dry Matter, Organic Matter and Fiber Digestion

True and apparent DM and OM digestion were not affected ($P > 0.05$) by BE inclusion (Table 3.2). Similarly, BE inclusion showed no effect ($P > 0.05$) on NDF and ADF digestion. These findings are in contrast to in vitro studies using whole hops by Narvaez et al. (2012) who demonstrated decreased true DM and NDF digestion when whole hops (72 g / kg of substrate) were included in long term fermentations with ruminal microbes using the Rusitec system. When similar concentrations of whole hops were added to batch culture incubations with ruminal microbes, Narvaez et al. (2011) observed a decrease in true DM disappearance when substrate was either barley grain or one of three feedlot diets with varying forage content. It is important to note that both studies by Narvaez et al. (2011, 2012) used whole hops, therefore, the confounding effects of additional fermentable material and plant secondary metabolites (condensed tannins) cannot be completely ruled out due to difficulty in achieving a proper control treatment. Narvaez et al. (2011) did include polyethylene glycol to inactivate condensed tannins (Jones and Mangan, 1977).

Hop extract preparations have demonstrated some moderate effects on digestion in in vitro rumen microbe fermentations. Narvaez et al. (2013) observed a reduction in true

DM disappearance in batch culture when α and β-acids were included at 22.5 and 30 ug/mL of inoculum (1.8 and 2.4 μg/kg substrate). Extract preparation differed, and inclusion level was far lower than the present study. In vivo experimentation with steers demonstrated no effects of BE or pelleted hops on DM, NDF and ADF digestibility (Schmidt et al., 2006; Wang et al., 2010), which was attributed to low β-acid inclusion rates (< 80 mg/kg diet DM for both studies), and instability of the hop compounds in feed.

Volatile Fatty Acid Production

Beta-extract inclusion had no effects ($P > 0.05$) on VFA concentrations in the fermenters (Table 3.3).Total VFA concentration (m*M*) was not affected by treatment, similar to the findings of Narvaez et al. (2013). In contrast to the findings of other studies using BE in pure and batch culture (Flythe and Aiken, 2010; Narvaez et al., 2013), A:P ratio was not affected by BE inclusion in the diet. Narvaez et al. (2011) reported that the effect of hops on lowering the A:P ratio in vitro was more pronounced in diets containing high concentrate *vs*. forage (86 *vs*. 40 % of diet DM as barley grain). In contrast, Wang et al. (2010) reported a more pronounced A:P response diets high in fiber *vs.* starch (40 *vs*. 86 % of diet as barley grain) were fed to feedlot cattle, although it was concluded that confounding effects of condensed tannins in BE could have been responsible for the differing results. Total VFA concentrations were within the range of previous continous culture studies conducted using lactating dairy rations (Mansfield et al., 1995; Castillejos et al., 2007).

Nitrogen Metabolism

Beta-extract inclusion level had no influence ($P > 0.05$) on fermenter nitrogen metabolism (Table 3.4). This is in contrast to Flythe (2009), where hyper ammonia producing bacteria (HAB) were shown to be sensitive to BE in pure and co-culture. In vivo research however has also shown no effects ($P > 0.05$) of hops on ruminal NH_3-N concentrations (Drouillard et al. 2009). Disagreement of effects on rumen nitrogen metabolism could be due to the influences of protozoa. Pure and co-cultures do not have protozoa present, and protozoal persistence in continuous culture systems is poor (Mansfield et al., 1995). Protozoa are known to affect nitrogen metabolism in the rumen through sequestration of microbial N (Koenig et al., 2000).

Dietary insoluble protein (DIP) was calculated at 61.5% of dietary CP as determined using NRC (2001) equations. Digestion of CP in the current study was consistent with previous research in continuous culture using diets with similar DIP values (Hussein et al., 1991; Griswold et al., 2003). Mansfield et al (1994) reported lower CP degradation values when DIP was increased from 50 to 70%. Efficiency of microbial crude protein synthesis was also within normal ranges (25-30 g/kg of OM truly digested) due to relatively similar levels of OM digestion and CP degradation (Mansfield et al., 1994; Miller-Webster et al., 2002).

Fermentation pH

BE inclusion tended to exert modest linear effects on fermentation pH (Table 3.5.) There were trends (P=0.09) for fermentation pH mean and max to increase with BE inclusion. Time spent above pH 6.2 tended (P = 0.08) to increase with greater BE

inclusion, while time spent between pH 5.8 and 6.2 tended ($P = 0.07$) to linearly decrease. Interestingly, time spent below pH 5.8 also tended ($P = 0.06$) to increase linearly with BE inclusion, indicating greater of variability of BE fermentation pH over time. These modest effects on fermentation pH are likely not of biologic importance and tendencies may be present simply due to high statistical power associated with the experimental design. Previous work with rumen microbes in the in vitro Rusitec system did not observe any effects of whole hops on fermentation pH (Narvaez et al., 2012). Al-Mamun et al. (2001) also reported no effects of spent hops when fed to sheep in sheep. Other studies with hops in batch culture studies rarely report pH because it is not often biologically relevant due to high buffering capacity needed to offset fermentation acid accumulation (Goering and Van Soest, 1970). Therefore comparison of fermentation pH between batch and continuous culture is not appropriate.

Microbial Adaptation

One possible explanation for the lack of effects of BE in continuous culture is the concept of microbial adaptation to β-acids. The ability of microbial populations to adapt to antimicrobial compounds is well documented (Lohner and Blondelle, 2005; Delcour, 2009).While in vitro batch culture studies often show strong effects, these systems typically use short term incubations (< 48 hours), thus limiting the time for ruminal microbes to adapt. The present experiment used a 7 day adaptation period to allow for stabilization of the continuous culture system. During this time, BE was administered via the buffer. A series of in vitro and in vivo experiments by Castillejos et al. (2007) demonstrated that effects of selective antimicrobial compounds in continuous culture fermenters can be dependent on adaptation time and donor cow diet. Authors suggested

that 6 d of adaptation were enough to reduce effects on VFA production and 4 weeks of adaptation were needed to observe effects on NH_3-N in the rumen (Castillejos et al. 2007). Pure culture studies have shown that ionophore resistance, specifically monensin, is largely mediated by presence of carbohydrate presence on the outer surface of the cells, as is the case with monensin-resistant *P. bryantii* B14 (Callaway and Russell, 1999). These considerations may be an explanation for limited effects of hops in the present study however data supporting this concept was not collected.

Differences Between In Vitro Models

It is important to note that this is the first known continuous culture evaluation of BE and to recognize relative differences of batch and continuous culture when comparing studies of hops and hop extracts. Availability of nutrients for fermentation differs substantially by culture method. Batch cultures have an inoculum to substrate ratio that is typically on an order of magnitude lower than that of the rumen, and substrate to buffer ratios are also lower due to concerns of end product accumulation (Goering and Van Soest, 1970). These combined differences result in batch culture incubations with 1/5 to 1/10 the microbial density per unit of substrate compared with continuous culture (Herbert et al., 1956; Hespell and Bryant, 1979). Additionally, comparable doses of BE may not be attained due to calculation methods used for in vitro experiments. Russell (2002) argued that physiologic dose may be exceeded when simple mathematics are used to calculate doses of antimicrobial compounds in batch culture because adjustments for dilution rate, bacterial density and binding of monensin to feed particles are not considered (Russell, 2002). In the current study, dosage was calculated to be within the range of previous batch culture studies; however the ratio of compound to bacteria in

those studies could have been much greater, resulting in an increased sensitivity to antimicrobial influences (Nagaraja and Talyor, 1987). The concept of increased sensitivity is also illustrated with the greater aerobic toxicity in batch culture, requiring the researcher to master anaerobic techniques when handling inoculum (Johnson et al., 1958). Ultimately, inherent differences between culture methods may partially explain the reason for positive effects of hops in pure and batch culture, and little to no effects observed with in vivo studies and the current study.

CONCLUSIONS

Inclusion of hop BE in continuous culture of rumen microbes produced no biologically significant alterations in fermentation parameters. Ingredient digestion, VFA production, nitrogen metabolism and fermentation pH parameters were within ranges of previous continuous culture studies using similar diets (Hannah et al., 1986; Mansfield et al., 1995, Calsamiglia et al., 1995). It is possible that ruminal microbes were able to adapt to protonophoric properties of BE, however no evidence was provided to support this theory, and further work investigating this concept is needed.

Table 3.1. Ingredient and chemical composition of experimental diet

Item	Composition[1]
Feed composition	
Corn silage	44.2
Alfalfa hay	13.5
Ground corn	12.6
Protein mix[2]	11.0
Corn gluten feed	9.6
Cottonseed	5.6
Liquid vitamin and mineral supplement[3]	3.4
Chemical composition	
Crude protein	17.5
Soluble nitrogen (% of total N)	38.5
NDIN (% of total N)	11.7
ADIN (% of total N)	5.3
NDF	31.5
ADF	17.1
Lignin	3.2
Ash	7.4
Starch	26.4
Sugar	5.4
NFC	41.3
Crude fat	4.4
TDN	73.5
$NE_{L,}$ 3X (Mcal/kg DM)	1.73

[1]Composition as % of 100°C DM unless otherwise noted.

[2]Composition (DM basis): canola meal, 28%; soybean meal, 22%; treated soybean meal, 15%; dried distillers grains, 13%; ground corn grain; 5%; calcium carbonate: 5%, blood meal, 3.5%; sodium bicarbonate, 3.5%; potassium carbonate, 3%; trace minerals, 2%.

[3]Supplement contained (DM basis): Ca, 49 g/kg; P, 11.7 g/kg; NaCl, 101 g/kg; K, 34 g/kg; Mg, 7.2 g/kg; S, 5.5 g/kg; Mn, 1237 mg/kg; Cu, 382 mg/kg; Se, 8.6 mg/kg; Zn, 1813 mg/kg; vitamin A, 171 IU/kg; vitamin D, 34 IU/kg; vitamin E, 706 IU/kg.

[4]Estimated from NRC (2001) equations.

Table 3.2. Effects of increasing levels of hop beta-extract on DM, OM and fiber digestion in continuous culture

	Treatment[1]				SEM[2]	*P*[3]	
Item, g/100g	**CON**	**LOW**	**MED**	**HIGH**		**Linear**	**Quadratic**
DM, apparent	40.4	37.5	38.1	38.1	1.9	0.50	0.49
DM, true[4]	58.1	55.7	55.0	56.3	2.7	0.61	0.49
OM, apparent	40.0	38.1	38.3	38.3	1.8	0.56	0.62
OM, true[4]	55.4	54.1	52.9	54.0	2.4	0.64	0.63
NDF	43.8	39.3	39.8	40.0	3.4	0.49	0.50
ADF	42.6	35.6	37.9	41.0	4.9	0.91	0.32

[1]CON: 0 mg β-acids/kg diet DM; LOW: 600 mg β-acids/kg diet DM; MED: 1200 mg β-acids/kg diet DM; HIGH: 1800 mg β-acids/kg diet DM.

[2]Standard error of the mean, n = 4 replicates per treatment.

[3]Probability corresponding to the null hypothesis with linear and quadratic contrasts.

[4]Corrected for bacterial contribution.

Table 3.3. Effects of increasing levels of hop beta-extract on VFA production in continuous culture

	Treatment[1]				SEM[2]	*P*[3]	
Item	**CON**	**LOW**	**MED**	**HIGH**		**Linear**	**Quadratic**
Total VFA, m*M*	116.0	119.8	116.9	122.2	4.5	0.45	0.86
Individual VFA, mol/100 mol							
Acetate	51.2	49.2	49.7	48.7	1.4	0.26	0.71
Propionate	29.1	32.3	32.7	32.4	1.9	0.25	0.38
Butyrate	13.7	12.5	12.3	12.2	1.1	0.37	0.62
Valerate	3.7	4.3	3.8	3.4	0.4	0.45	0.25
Isobutyrate	0.29	0.21	0.21	0.41	0.10	0.43	0.20
Isovalerate	0.39	0.17	0.18	0.29	0.11	0.59	0.16
Branched-chain VFA, m*M*	2.61	1.93	1.84	3.87	1.03	0.44	0.21
A:P Ratio	1.77	1.53	1.53	1.56	0.14	0.32	0.34

[1]CON: 0 mg β-acids/kg diet DM; LOW: 600 mg β-acids/kg diet DM; MED: 1200 mg β-acids/kg diet DM; HIGH: 1800 mg β-acids/kg diet DM.

[2]Standard error of the mean, n = 4 replicates per treatment.

[3]Probability corresponding to the null hypothesis with linear and quadratic contrasts.

Table 3.4. Effects of increasing levels of hop beta-extract on nitrogen metabolism in continuous culture

Item	Treatment[1]				SEM[2]	*P*[3]	
	CON	LOW	MED	HIGH		Linear	Quadratic
NH_3-N, mg/dL	6.9	5.1	6.7	7.5	0.8	0.38	0.13
N flow, g/d							
NH_3-N	0.17	0.13	0.17	0.18	0.02	0.56	0.18
Non NH_3-N	2.17	2.25	2.20	2.17	0.06	0.82	0.40
Microbial-N	1.15	1.17	1.10	1.13	0.06	0.61	0.89
Dietary-N	1.02	1.08	1.10	1.03	0.09	0.87	0.51
CP degradation, %	62.0	58.9	58.1	60.8	3.6	0.84	0.46
EMPS[4]	28.2	29.8	28.5	28.6	1.2	0.98	0.56

[1]CON: 0 mg β-acids/kg diet DM; LOW: 600 mg β-acids/kg diet DM; MED: 1200 mg β-acids/kg diet DM; HIGH: 1800 mg β-acids/kg diet DM.

[2]Standard error of the mean, n = 4 replicates per treatment.

[3]Probability corresponding to the null hypothesis with linear and quadratic contrasts.

[4]EMPS: efficiency of microbial protein synthesis (g of microbial N/kg of OM truly digested).

Table 3.5. Effects of increasing levels of hop beta-extract on fermentation pH in continuous culture

Item	Treatment[1]				SEM[2]	*P*[3]	
	CON	LOW	MED	HIGH		Linear	Quadratic
Mean pH[4]	5.73	5.76	5.79	5.81	0.04	0.09	0.87
Minimum pH[4]	5.69	5.69	5.71	5.73	0.03	0.21	0.89
Maximum pH[4]	5.78	5.82	5.87	5.88	0.04	0.09	0.78
Time below pH 5.8[5]	1.3	2.5	24.4	117.3	40.6	0.06	0.28
Time between pH 5.8 and 6.2[5]	3654.2	3069.0	2652.2	2213.3	532.9	0.07	0.89
Time above pH 6.2[5]	659.6	1243.5	1638.4	1984.4	519.3	0.08	0.82

[1]CON: 0 mg β-acids/kg diet DM; LOW: 600 mg β-acids/kg diet DM; MED: 1200 mg β-acids/kg diet DM; HIGH: 1800 mg β-acids/kg diet DM.

[2]Standard error of the mean.

[3]Probability corresponding to the null hypothesis with linear and quadratic contrasts.

[4]Analyzed as repeated measures with 1 observation/fermenter/hour during 3 consecutive sampling days.

[5]Total minutes during 3 consecutive days, n = 4 replicates per treatment.

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