Effect of yeast, protected minerals and bismuth subsalicylate on in vitro fermentation by rumen microbes

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Dedication

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Chapter 1 : Review of literature

1. EFFECT OF LIVE YEAST ON RUMEN METABOLISM

1.1. Introduction

The term "probiotics" was defined by Fuller (1989) as "live microbial feed supplements which beneficially affect the host animal by improving its microbial balance". Several commercial formulations of probiotics including *Lactobacillus acidophilus*, lactic acid producing and utilizing bacteria, propionibacteria, and yeasts are commercially available (Jouany and Morgavi, 2007). Currently, more than 1000 strains of *Saccharomyces cerevisiae* are listed in the American Type Culture Collection catalogue (1990). Among them, many different products are marketed for ruminant nutrition, most of them based on the presence of *S. cerevisiae* (SC) (Oeztuerk et al., 2005). Products containing highly concentrated dry yeast cells (>10 billion cfu/g) without the culture medium are usually referred to as active dry yeast (ADY). Conversely, yeast culture products (YC) contain live yeast cells and the fermentation medium on which they were grown. In addition, commercial preparations containing the inactivated SC are available as feed ingredients (Lynch and Martin, 2002; Fonty and Chaucheyras-Durand, 2006), but because they have no biological activity related to the presence of live organisms, they will be excluded from this review.

1.2. Effects of yeast on rumen fermentation

Different mechanisms of action of yeasts in the rumen are summarized in Figure 1.1 (Jouany and Morgavi, 2007). However, a comprehensive understanding of those mechanisms and their interactions is not totally elucidated (Oeztuerk and Sagmanligil, 2009; Hristov et al., 2010). It has been suggested that an increase in rumen bacterial population in animals receiving SC is central in the action of yeast in the rumen, producing an increase in fiber digestibility and microbial flow to the duodenum (Newbold et al., 1996). Yoon and Stern (1996) reported that yeast supplementation increased proteolytic bacteria population. In addition to bacteria, Chaucheyras et al. (1995a) found that addition of yeast stimulated zoospore germination of *Neocallimastix frontalis*, increasing cellulose degradation. The proposed mechanism for increasing fungi and bacteria populations is apparently related to the contribution of growth factors (B-vitamins, AA, organic acids) obtained with the addition of yeast (Wiedmeier et al., 1987).

Yeast are aerobic organisms that cannot survive for long period in the rumen. Chaucheyras-Durand et al. (1998) established that once the yeast product is ingested, steady concentrations are found in rumen during the next 24 h without significant growth, and the concentration decays to undetectable levels in 4 to 5 days if yeast is not feed regularly. While the rumen is traditionally considered an anaerobic ecosystem, dissolved O_2 can be detected. As an example, a daily turnover of as much as 16 l O_2/d has been calculated in sheep, entering the rumen during feed

and water intake, salivation, rumination and diffusion trough the rumen wall. The role of live yeast in O_2 removal from the rumen has been previously established and is partially responsible for the probiotic activity exhibited by yeast (Newbold et al., 1996). In the rumen, the partial pressure of O_2 helps to regulate the lactate-propionate equilibrium according to the following equation:

$$C_3H_6O_3 \text{ (lactate)} \leftrightarrow C_3H_6O_2 \text{ (propionate)} + \frac{1}{2} O_2$$

When partial pressure of O_2 is decreased, formation of propionate from lactate is favored. On the contrary, when O_2 pressure is increased, accumulation of lactate may occur. According to Jouany and Morgavy (2007), this mechanism provides an explanation to pH stabilization accomplished by yeast when ruminants are feed a diet rich in highly fermentable carbohydrates. However, other mechanisms have been suggested to explain the effect of yeast in ruminal pH. Callaway and Martin (1997) proposed the release of small peptides and malate from yeast as a mechanism stimulating the lactate uptake by *Megasphaera elsdenii* and *Selenomonas ruminantium*. Plata et al. (1994) found an increase in total protozoa population in animals receiving yeast. According to Chamberlain et al. (1983), conversion of lactate to propionate and butyrate increases in the presence of protozoa. Protozoa have been identified as important agents causing death of *Streptococcus bovis* (Jarvis, 1968), responsible for L-lactate production during rapid growth (Russell and Baldwin, 1979).

Several *in vivo* and *in vitro* studies showed a benefit of SC in stabilization of rumen pH. Thrune et al. (2009) reported an increase in mean, minimum and maximum rumen pH of 0.2 units following administration of 0.5g ADY/cow/d (CNCM-1077, Levucell SC20 SC, Lallemand Animal Nutrition) to late lactation dairy cows receiving a 60% forage and 40% concentrate diet and housed in tie-stalls. Similarly, Bach et al. (2007) reported an increment in average rumen pH and average maximum pH of 0.5 units, and average minimum pH of 0.3 units, when 5.0 g/d of ADY (CNCM I-1077 (Levucell SC2, Lallemand, Toulouse, France) were provided to lactating dairy cows maintained in loose-house conditions. A comparable increment in mean rumen pH was reported recently by Křížová et al. (2011) in dry Holstein cows supplemented with 3.0 g ADY (BIOSAF Sc 47, Lesaffre, France) twice daily. Using Fresian steers, Williams et al. (1991) found a reduction in rumen lactate concentration following supplementation of 7.5 g YC/d. According to Nisbet et al. (1991), and Callaway and Martin (1997), this effect is mediated by the ability of SC to stimulate uptake of lactate by *Selenomonas ruminantium*. However, while the increase in ruminal pH is a relatively consistent finding in the literature, a related decrease in lactic acid concentration is more ambiguous. In a meta-analysis conducted by Desnoyers et al. (2009) on 157 experiments and 376 treatments including the use of SC, only a trend for reduction in lactate was found, suggesting that yeast may affect rumen pH due to their ability to increase buffer capacity of rumen liquor as previously suggested (Giger-Reverdin et al., 2004).

In opposition to relatively constant findings in rumen pH, effects of SC on rumen VFA are generally weak and non significant (Yoon and Stern, 1996; Enjalbert et al., 1999; Galip, 2006). While the meta-analysis conducted by Desnoyers et al. (2009) showed an increase in VFA concentrations by addition of SC, there was only a 2% difference between treated and non-treated animals. A higher increment in total VFA (+5.4%) was reported by Robinson (2002) after reviewing 20 experiments involving the use of SC. However, no effect of SC on total VFA was found by Sauvant et al. (2004) and Lescoat et al. (2000) after reviewing 78 experiments and 40 manuscripts on use of SC, respectively. In other studies (Tripathi et al., 2008; Thrune et al., 2009), addition of SC resulted in a trend for reduction in total VFA. Apparently, the effect of SC on rumen VFA is related to dietary composition. Using 3 different forage:concentrate ratios (90:10, 65:35 and 40:60) , Chademana and Offer (1990) observed that SC increased VFA more when dietary concentrate proportion was highest. According to Erasmus (1992), the effect of SC on rumen VFA is related to their ability to increase propionogenesis. Conversely, Chaucheyras et al. (1995b) found an *in vitro* increase in acetogenesis and a shift in H_2 deposition from CH_4 to acetic acid following addition of live or autoclaved SC to purified cultures containing acetogenic bacteria and methanogenic archea. According to the authors, the presence of a non identified heat-resistant factor could be implicated in this process. Similarly, Lila et al. (2006) found a small decrease in CH_4 production in batch cultures inoculated with high doses of SC (up to 1 mg/mL). In addition, a decrease in CH_4 was found by Mutsvangwa et al. (1992) using rumen fluid from bulls

supplemented with SC. However, when yeast culture was added (15 mg YC/g of DM) to a rumen simulation technique apparatus (RUSITEC), inconclusive responses on CH_4 were obtained. In agreement, *in vivo* results of Mathieu et al. (1996) and McGinn et al. (2004) showed no benefits in using SC to control CH_4 emissions from sheep and steers.

1.3. Effects of yeast on rumen nitrogen metabolism

In the rumen, most dietary proteins are degraded by the microbial population to peptides, free amino acids and ammonia. These end-products can be utilized for protein synthesis by rumen microbes. However, when dietary protein degradation is faster than microbial nitrogen utilization, the resulting excess of nitrogen and consequent loss of ammonia from the rumen occurs in a process referred to as ammonia overflow (Broderick, 1996). The metabolic fate of this ammonia excess is to be excreted in urine, in the form of urea which is converted to N_2O, a volatile compound with a global warming potential 310-fold that of CO_2 and more than 14-fold that of methane (Mitloehner et al., 2009). Data on the effects of live yeast on microbial metabolism of N in the rumen are inconclusive (Fonty and Chaucheyras-Durand, 2006; Chaucheyras-Durand et al., 2008). An *in vivo* study of Kumar et al. (1994) showed a decrease in rumen ammonia in buffalo calves receiving 5 g/d of SC. Similarly, a reduction in rumen ammonia N was observed in naturally raised and gnotobiotically-reared lambs receiving the microbial additive SC (CNCM I-1077), and it was hypothesized that ammonia utilizing bacterial populations were more

active in the presence of live yeast (Chaucheyras-Durand and Fonty, 2001; Chaucheyras-Durand and Fonty, 2002). However, only trends for a reduction in ammonia N were found in early studies from Erasmus et al. (1992) and from Harrison et al. (1988).

In a preliminary study conducted by Chaucheyras-Durand et al. (2005), the proteolytic activity of *Prevotella albensis* and *Butyrivibrio fibrisolvens*, two major species among the proteolytic rumen bacteria, was reduced by addition of autoclaved SC but not when live SC were added. The authors hypothesized that small specific peptides released during yeast autoclaving could alter the action of bacterial enzymes, as an explanation for the observed effect. Sniffen et al. (2004) summarized the effect of SC CNCM I-1077 on rumen nitrogen metabolism in 14 experiments conducted in dairy cows. The authors concluded that the effect of SC was related to the dietary level of soluble N (ammonia, amino acids and peptides), resulting in lower N losses when an adequate balance between soluble N and carbohydrates was provided. However, no effect of SC in ammonia N concentration was reported *in vitro* or *in vivo* in early lactating (Yoon and Stern, 1996; Putnam et al., 1997), late lactating (Thrune et al., 2009) and non-lactating (Enjalbert et al., 1999) dairy cows. While most researchers hypothesize that the large variations in effects of SC are related to dietary composition, Jouany (2001) proposed a role of particle structure of the diet. According to the author, yeast survive in the rumen in a "micro-consortium structure" located within and immediately around freshly ingested solid particles, in close association with oxygen-containing bubbles trapped in dietary material. At

present, the impact of dietary particle size on survival of SC is not documented. Moreover, the impact of micro-consortium structures is lacking in *in vitro* models such as continuous culture fermenters, and its relationship with SC effects is not established. For this reason, Chaucheyras-Durand et al. (2008) suggested that more research is needed to better understand how dietary factors influence the impact of yeast on rumen microbial metabolism.

1.4. Animal responses to yeast supplementation

Beneficial effects of adding yeast to diets of ruminants have been extensively documented. In dairy cows, addition of SC is usually associated with an increase in DM intake and OM digestibility (Williams et al., 1991; Desnoyers et al., 2009) and the consequent impact on milk production. In a review of 22 studies involving more than 9039 dairy cows (Dawson, 2000), supplementation with SC resulted in an average increase in milk production of 7.3%, but with a large variation, that ranged from 2 to 30%. In a study conducted with Jersey cows, Dann et al. (2000) observed an increase in DMI during the last 7 days pre-partum and the following 42 days post-partum. Similarly, postpartum DMI increased with Holstein cows that received a DFM preparation containing SC (Nocek et al., 2003), probably related to an increase in some specific dietary compounds (Nocek et al., 2002). However, in studies conducted by Robinson and Garrett (1999), and by Swartz et al. (1994) on uniparous and multiparous Holstein cows, no differences in DMI were obtained.

Besides an increase in DMI or OM digestibility, other mechanisms have been suggested as partially responsible for the improvement in productivity attained by the addition of SC to the ration of dairy cows. Lesmeister et al. (2004) observed an increase in rumen papillae length and width in Holstein calves receiving SC. In addition, an improvement in health status of was observed (Magalhães et al., 2008) when yeast culture was supplemented to the diet of Holstein calves. In humans and rats, ingestion of *Saccharomyces boulardii* has been associated with increased activity of jejunal disaccharidases (i.e., sucrase) (Buts et al., 1986). In addition, during its intestinal transit, *S. boulardii* secretes polyamines, mainly spermine and spermidine that regulate gene expression and protein synthesis (Buts and De Keyser, 2006). However, no information regarding the relevance of this mechanism on intestinal function of ruminants is available.

While some improvements in health and productive performance have been reported in beef cattle (Mutsvangwa et al., 1992), the benefits of dietary supplementation with SC appears less evident in beef than in dairy cattle. Beauchemin et al. (2003) reported no differences in rumen fermentation and rumen pH in cannulated steers receiving SC alone or in combination with bacterial probiotics. Similarly, Gomes et al. (2009) only found an increase in carcass dressing percentage of Nellore steers, but addition of SC to the diet did not affect longissimus area, subcutaneous fat thickness, meat color, carcass fat content or carcass pH. Furthermore, Emanuel et al. (2007) found an increase in acute phase proteins, (serum amyloid A, lipopolysaccharide binding protein, haptoglobin, and $alpha_1$-acid

glycoprotein) indicative of inflammatory response in steers receiving SC in combination with *Enterococcus faecium*. While the reason for these findings is unclear, the authors suggest that it may be related to lysis of coliform bacteria when yeast was added to the diet.

The reason for such a disparity in animal response has been related to variation in dietary characteristics (Arcos-García et al., 2000; Castillo et al., 2006), interactions between dietary compounds and yeast (Fiems et al., 1993), differences between yeast strains, and experimental conditions (Newbold et al., 1995). For these reasons, Tripathi et al. (2011) suggest that further studies with more varities of yeast strains are required for their suitability to develop promising microbial additives.

In conclusion, addition of SC to the diet of ruminants provides some benefits mostly related to pH stabilization and direct or indirect reduction in lactic acid concentrations. However, highly variable responses are obtained regarding rumen nitrogen metabolism. While some studies showed an improvement in animal production, addition of SC seems to be more effective with dairy cows than with feedlot animals. The high diversity of yeast strains available in the market and the unknown interaction with dietary characteristics ensures the need for further research.

2. ROLE OF ZINC, MANGANESE, AND COPPER, IN RUMEN METABOLISM

2.1. Introduction

At present time, 111 chemical elements are officially accepted by The **International Union of Pure and Applied Chemistry** (Karol et al., 2003). Out of them, 93 are classified as naturally occurring, and approximately 50 have been identified as useful to sustain a normal health status in mammals. In addition to the six core elements (C, H, N, O, S and P) that make up nucleic acids, proteins, carbohydrates, and lipids and thus the bulk of living matter (Wolfe-Simon et al., 2010), many other elements are essential to the nutritional requirements of superior animals. Minerals required in relatively large quantities (g/d) are named "macro" minerals. In opposition, minerals required in mg or μg amounts are referred to as "micro" or "trace" minerals (NRC, 2001). Minerals are usually classified in four broad groups according to their physiological roles:

- Structural: minerals forming structural components of the body organs and tissues which include Ca, P, Mg, Fl and Si in bones and teeth and P and S in muscle proteins.
- Physiological: minerals occurring on body fluids and tissues as electrolytes, involved in the maintenance of osmotic pressure, acid base-equilibrium, membrane permeability and tissue stimuli (e. g. Na, K, Cl, Ca and Mg in blood and cerebrospinal fluid).

- Catalytic: minerals acting as catalysts in enzymatic systems, as integral compounds in the structure of metalloenzymes or as cofactors required for enzymatic activation (e. g. Fe, Cu, Zn, Mn, and Se involved in cytochromes, ceruloplasmin, carbonic anhydrase, pyruvate carboxylase, and glutathione peroxidase, respectively).
- Regulatory: minerals involved in regulation of cell replication and differentiation (e. g. Ca, in signaling transduction; Zn, in DNA transcription).

However, this classification is arbitrary and not exclusive, since the same element can fulfill more than a single function (Underwood and Suttle, 2001).

Ideally, dietary supply of minerals to livestock must be sufficient to ensure the maintenance of body reserves, and to provide adequate concentrations in edible products. However, in some occasions, drinking water can be an exceptionally rich source of minerals, sometimes responsible for mineral toxicity (Suttle, 2010). The accomplishment of an adequate supply of minerals is especially challenging since requirements for most minerals are not constant, but affected by physiological factors including genetics, age, sex, type of production (maintenance, growth, reproduction, and lactation), and level of production (Spears, 2002).

2.2. Availability of minerals to animals

The evaluation of feedstuffs and mineral supplements for the host animal depends not only on the mineral content in the sources but also on the potential

availability and absorption of the minerals from the gastrointestinal tract and the mineral's utilization by the animal tissues (Ammerman, 1995). However the potentially absorbable fraction of a mineral is greatly influenced by several factors including age and species of animal, intake of mineral relative to amount required, chemical form of the mineral, and the amount and proportions of other dietary compounds involved in specific interactions with the mineral (Hazell, 1985). Livestock diets are often supplemented with trace minerals in the form of inorganic salts, usually oxides, chlorides, sulfates and carbonates. In recent years there has been considerable interest in the use of organic trace minerals in ruminant diets (Rabiee et al., 2010). According to the Association of American Feed Control Officials (AAFCO, 2000), organic trace minerals in the United States are available in one of the following forms:

- *Metal Proteinate*: the product resulting from the chelation of a soluble salt with amino acids and/or partially hydrolyzed protein. e.g. Cu proteinate, Zn proteinate, Co proteinate and Mn proteinate.
- *Metal Amino Acid Chelate*: the product resulting from the reaction of a metal ion from a soluble metal salt with amino acids with a mole ratio of one mole of metal to one to three (preferably two) moles of amino acids forming coordinate covalent bonds. For example, Cu-lysine-sulfate is the sulfate salt of 2:1 molar ratio of L-lysine and Cu. Similarly Cu methionine-bisulfate is the bisulfate salt of a 1:1 molar ratio of DL-methionine and Cu. Other commercially available metal amino acids are Cu amino acid chelate, Zn

amino acid chelate and Mn amino acid chelate in addition to chelates for macro minerals like Ca and Mg.

- *Metal Amino Acid Complex*: the product resulting from complexing a soluble metal salt with an amino acid. Commercially available metal complexes are Zn-methionine, Zn-lysine, Mn-methionine, Fe-methionine and Cu-lysine.
- *Metal Polysaccharide Complex*: the product resulting from complexing a soluble salt with a polysaccharide solution declared as an ingredient of the formulation (e.g. Cu polysaccharide complex, Zn polysaccharide complex, etc).

While the effectiveness of organic minerals for ruminants has been strongly criticized (Suttle, 2010), Roy and Misger (2008) established that for chelation to be effective, the chelating agent should have a stronger stability for the metal than the metal binding substances in feed, but smaller stability constant than the tissue system where the metal is required. In addition, other factors including metal ion equilibria, kinetic factors, pH gradients and redox equilibrium (in case of redox active metals such as Cu^{2+}), may also affect the uptake mechanism of metal ions (Hynes and Kelly, 1995). Several studies have been conducted to address the benefits of including organic sources of minerals on the productive performance of ruminants. However, in many cases a combination of several organic elements has been used, making difficult to assess which one is responsible for the effects noticed, as discussed later.

The aim of this review is to explore the factors involved in bioavailability of Zn, Mn and Cu at the gastrointestinal tract, affecting the mineral status of the host animal. In addition, the role of Zn, Mn and Cu on ruminal physiology is presented.

2.3. Zinc

2.3.1. Interaction of zinc with dietary factors

Early studies from Miller (1970) showed that in ruminants, the percentage of dietary Zn absorbed decreases as dietary Zn increases. Ansari et al. (1976) found a linear increment in endogenous fecal loss of Zn when rats were feed increasing levels of dietary Zn ranging from 0 to 8400 ppm. Similarly, in a study conducted in growing pigs receiving ^{67}Zn, the addition of phytase increased Zn absorption but also resulted in higher endogenous fecal loss (Chu et al., 2009). Concurrently, reductions of urinary and fecal Zn excretion by 48% and 46%, respectively were found in humans when dietary Zn was reduced from 85 to 12 μmol/d. Despite the influence of dietary levels, the requirement of Zn appears to be affected by other dietary factors. However, all of the factors and interactions that play a role on Zn bioavailability are not clearly defined (Spears, 2003). According to Baker and Ammerman (1995), the two major dietary factors affecting Zn bioavailability are the presence of organic chelating agents and the interaction with metallic ions, with Cu and Ca being the most important antagonists.

2.3.2. Role of zinc on rumen fermentation

Early studies from Sonawane and Arora (1976) showed that *in vitro* microbial protein synthesis was increased together with a reduction in NH_3-N concentration when rumen fluid was incubated with additional Zn as $ZnCl_2$ or $ZnSO_4$. According to the authors, this response is due to an effect of Zn in increasing microbial enzymatic activity. However, further studies from Bonhomme et al. (1979) showed differences in the behavior of rumen microbial populations in the presence of Zn. While protozoa easily incorporated Zn and were tolerant to high Zn concentrations (25 μg/mL), cellulose degradation from rumen bacteria was deeply depressed, along with bacterial urease activity. In partial agreement, Martinez and Church (1970) found that addition of 5 μg Zn/mL of rumen fluid increased cellulose digestion by 24%, but addition of 20 μg/mL of Zn depressed it by 31%. Arelovich et al. (2000) found that low supplementation levels (10 to 15 ppm Zn in incubation fluid) inhibited *in vitro* urea hydrolysis and retarded ammonia accumulation. Similarly, when Zn was added *in vivo* at 250 ppm Zn on DM basis, molar proportion of propionate was increased with the consequent decrease in acetate:propionate ratio, and rumen ammonia was decreased due to microbial urease inhibition. However, when Zn was added to achieve a level of 470 ppm on DM basis, a tendency for depressed DM digestibility was noticed. In addition, Spears et al. (2004) found a decrease in total VFA when Zn was provided to steers as Zn methionine or Zn glycine compared with $ZnSO_4$ at concentrations closer to physiological levels (20 ppm). In the same study, molar proportion of propionate was increased by Zn methionine and that of butyrate was

decreased, with the consequent reduction in the acetate:propionate ratio. The authors conclude that supplementation of Zn methionine may alter ruminal fermentation.

While these differences can be partially explained by the dose of Zn and the fermentation substrate used by the different authors, an alternative mechanism is proposed by Eryavuz and Dehority (2009), who found that addition of 50 μg/mL of Zn to *in vitro* incubations decreased cellulose digestion at 24 h, but not at 48 h, resulting in an overall decrease in the rate, but not the extent of digestion. The authors conclude that the initial decrease in cellulose digestion might be related to a direct effect of Zn on inactivation of bacterial cellulase, since heavy metal salts can precipitate and denature soluble proteins and enzymes. However, enough cellulase activity may be present to overcome those negative effects of high Zn concentrations. In addition, the accumulation of Zn in bacterial wall (Bonhomme, 1990) might be affecting the adhesion of microbial cells to cellulose particles, a limiting step in cellulose fermentation as previously established (Pell and Schofield, 1993).

2.3.3. Zinc availability from organic and inorganic sources

There is scientific evidence showing that organic Zn is metabolized differently than inorganic sources. In four consecutive studies conducted by Spears (1989), Zn was better retained when added as Zn methionine than ZnO in lambs and heifers. However, the observed improvement was not due to higher absorption but to a lower urinary Zn excretion in animals receiving Zn methionine, and only minor changes in

blood parameters were noticed. Similarly, in a study conducted by Mandal et al. (2008) in calves, organic or inorganic Zn supplementation did not affect concentration of serum enzymes (alkaline phosphatase, glutamate oxaloacetate transaminase, glutamate pyruvate transaminase and super oxide dismutase) or mean concentrations of different serum vitamins (retinol, ß-carotene, α-tocopherol) and hormones (triiodothyronine, thyroxin, insulin and testosterone). Furthermore, blood parameters from supplemented groups did not differ from unsupplemented controls. In a comparative study, Dorton et al. (2010) found no differences in liver Zn and plasma Zn concentration of steers receiving $ZnSO_4$ or Zn amino acid complex. However, confounding effects may account for these findings, given the differences in mineral status of the animals at the beginning of the study. In partial agreement, no differences in plasma Zn concentration of steers receiving $ZnSO_4$, Zn methionine complex or Zn glycine were found by Spears et al. (2004). Although large variations prevented from obtaining differences in absorbed or retained Zn, Zn glycine resulted in larger liver Zn concentrations. Conversely, using organic and inorganic Zn, Huerta et al. (2002) found higher concentrations of Zn in plasma of supplemented beef steers than in controls, despite the Zn source. Interestingly, in animals receiving an implant containing estradiol benzoate and testosterone propionate, weight gain resulted higher when Zn was supplemented as $ZnSO_4$ that of Zn propionate (Huerta et al., 2002). In a large production study including 250 dairy cows, only a tendency for improvement in milk production was obtained when Cu, Zn, Mn and Co as sulfates, were replaced by organic forms of the minerals. Liver

concentration of minerals was unaffected by mineral source, but supplementation with organic minerals resulted in increased milk solids and a decreased incidence of sole ulcers (Siciliano-Jones et al., 2008). In another large scale study conducted on 573 dairy cows (Nocek et al., 2006), supplementing 75% of the requirement of Zn as Zn methionine achieved the same hepatic Zn concentration than supplementing 100% of the requirement as $ZnSO_4$. Based on the lack of differences in health and productive performance the authors suggest that mineral content of liver is not an accurate predictor of cow's response to different sources and levels of trace minerals. In agreement, Siciliano-Jones et al. (2008) suggest that Zn, Mn, and Cu content of liver is a poor indicator of trace mineral status. According to Underwood and Suttle (2001), the supposed benefits of organic sources of Zn on Zn availability claimed in studies conducted in monogastrics cannot be adopted in ruminants since phytic acid, a major antagonist of Zn absorption, is largely hydrolyzed in the rumen. An additional difficulty in the evaluation of Zn availability from different sources relies on the fact that Zn is absorbed according to the animal needs and homoeostasis in ruminants is achieved primarily by control of intestinal absorption (Suttle et al., 1982).

2.4. Manganese

2.4.1. Interaction of manganese with dietary factors

According to the NRC (2001), there is no exact data regarding the maintenance requirements of Mn in dairy cattle. However, the coefficient of intestinal absorption

for Mn in adult cattle is known to be as low as 1% of ingested Mn or even lower (Hidiroglou, 1979; Spears, 2003), but the absorption in young calves is considerably higher (Howes and Dyer, 1971). Despite the generalized idea of poor Mn absorption, Underwood and Suttle (2001) suggested that this situation is partly a reflection of the substantial surplus of Mn provided by most practical rations, since higher coefficients of absorption were obtained when animals received diets marginal in Mn (Atkinson et al., 1993). For this reason, the NRC (2001) adopted a conservative coefficient of 0.75% for Mn absorption. In agreement, Sansom et al. (1978) reported a coefficient of 0.54% for Mn absorption in dairy cows.

The study of dietary factors influencing Mn bioavailability has received little attention, probably because Mn deficiency is not considered to be a major problem in ruminants (Spears, 2003). In addition, most of the information available has been generated in monogastric models. Henry et al. (1986) observed a 10 and 13% increase in kidney and bone Mn, respectively from chicks receiving 12 ppm of virginiamycin in the diet. In a further study (Henry et al., 1987), the addition of 4 ppm lincomycin resulted in higher concentrations of Mn in bone. However, while virginiamycin and other antibiotics are currently used as feed additives for ruminants (Salinas-Chavira et al., 2009), their role in Mn absorption in cattle remains unknown.

According to Hidiroglou (1979), the intestinal absorption of Mn is negatively affected by dietary levels of Ca and P. Similar results were found by Baker and

Oduho (1994), who found a 45% reduction in Mn in the tibia of chicks fed excess Ca and P. However, further evidence provided by Wedekind et al. (1991) indicated that while P has a negative effect on Mn absorption, no deleterious effects on Mn metabolism are obtained with excess of dietary Ca, but effects of Ca and P are difficult to distinguish since diets are usually enriched with both minerals to maintain a Ca:P physiological ratio (Suttle, 2010). There have been no reports relating Mn absorption with forage composition in ruminants, but phytate and fiber are known to be the main antagonists in monogastrics species including human (Davidsson et al., 1995), swine (Richards et al., 2010) and poultry (Ji et al., 2006). Through microbial activity, both phytates and fiber are broken down in rumen (Yanke et al., 1998). For that reason, Suttle (2010) suggests that Mn absorption in ruminants may not be affected by the presence of phytates, being higher than that usually reported for monogastrics.

2.4.2. Role of manganese on rumen fermentation

Limited information is available regarding the role of Mn in rumen fermentation. According to Spears and Hatfield (1978), Mn has a slight stimulatory effect on urease activity. Arelovich et al. (2000) reported a 6% increment in IVDMD when Mn was added to incubations at a dose of 100 ppm. In a previous study, Chamberlain and Burroughs (1962) found a reduction in cellulose digestion when Mn was omitted from *in vitro* incubations. However, Martinez and Church (1970) reported that cellulose digestion peaked at Mn concentrations of 10 to 20 ppm but

was completely abolished when Mn was added at 300 ppm. Using ^{54}Mn, Ivan (1979; 1981) observed that Mn accumulation was higher in rumen bacterial cell walls than in cytoplasm, and that the uptake was similar in bacteria and protozoa, but the biological implications of this fact are not established. Masters et al. (1988) fed ram lambs diets that contained from 13 to 45 mg of Mn/kg DM during 84 days. While the number of rumen bacteria was not affected by Mn, the large rumen bacteria (identified as those with a diameter of 12.9 to 16.2 μm) resulted lower with the lowest Mn intake and highest with dietary Mn provided at 30 mg/kg. This may be particularly relevant since large rumen bacteria contain more protein than small rumen bacteria (Czerkawski, 1976). However, despite this variation in microbial populations, no effect of Mn on DM digestibility was observed. Panggabean et al. (1984) suggested that sheep consuming a diet high in fiber and low in protein may respond to Mn supplementation in excess of 36 μg/g DM, but Mn requirements of the rumen microbes may be increased by the consumption of low quality roughages. In agreement, Durand and Kawashima (1980) suggest that the optimum content of Mn in the diet may be as high as 120 μg/g DM, on the basis of results from *in vitro* studies.

2.4.3. Manganese availability from organic and inorganic sources

The aim of mineral supplementation is to increase the biological availability of the target mineral(s), defined as the degree to which an ingested element is absorbed and can be utilized in metabolism by the animal (Forbes and Erdman, 1983). Different sources of Mn are currently available as supplements for animal

diets. Among the inorganic sources, the most commonly used include manganese carbonate ($MnCO_3$), hausmannite (Mn_3O_4), manganese oxide (MnO), manganese dioxide (MnO_2), manganite (Mn_2O_3), manganous chloride ($MnCl_2.4H_2O$) and manganese sulfate ($MnSO_4$) (Ammerman and Miller, 1972; Wong-Valle et al., 1989; McDowell, 1992; Underwood and Suttle, 2001). Conversely, sources of Mn classified as "organic" include Mn-methionine, Mn-proteinate and Mn-polysaccharide (DiCostanzo et al., 1986; Scheideler, 1991; Henry et al., 1992; Smith et al., 1995). Unfortunately, only few studies have been conducted comparing the relative bioavailability of Mn sources in ruminants fed physiological concentrations of Mn (Spears and Hansen, 2008). According to Kratzer (1986) some chelates and complexes may improve the mineral bioavailability above that of soluble inorganic forms, as later shown by Henry et al. (1992) in lambs comparing Mn-methionine with MnO. However, no differences were obtained in the same study when Mn-methionine was compared with $MnSO_4$. Similarly, Li et al. (2005) compared the bioavailability of Mn from different organic sources and $MnSO_4$ in broilers. The authors concluded that only organic Mn sources with moderate or strong chelation strength can provide higher relative bioavailabilities due to their ability to resist Ca antagonisms during the digestion process. In addition, studies conducted on female chicks showed an increase in Mn retention from a Mn-methionine chelate compared with MnO (Fly et al., 1989).

2.5. Copper

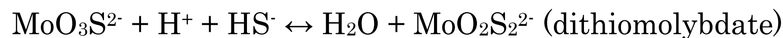
2.5.1. Interaction of copper with dietary factors

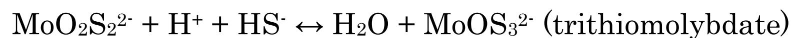
The amount of dietary Cu needed to supply Cu requirements for maintenance, growth and lactation varies with the age of the animal, the chemical form of dietary Cu and the presence of dietary substances interfering with Cu absorption (NRC, 2001). Compared with monogastrics where Cu is fairly well absorbed (30 – 75%), absorption in adult ruminants is low, ranging from 1 to 10% of dietary Cu (Underwood and Suttle, 2001; Linder, 2002). However, before developing a functional rumen, Cu absorption in lambs can be as high as 70-85% of the dietary supply (Suttle, 1975). The reason for this decrease in Cu absorption appears to be related to interactions occurring at the rumen environment, including the Cu-S-Mo (Dick, 1953; Ward et al., 1993; Suttle, 2010), Cu-S (Suttle, 1974; Arthington et al., 2002), and Cu-Fe (Jarvis and Austin, 1983; Mackenzie et al., 2008) antagonisms. More recently, results relating high levels of dietary Mn with Cu deficiency have been reported (Legleiter and Spears, 2007; Hansen et al., 2009).

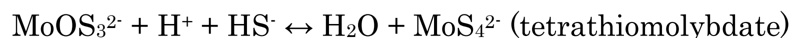
Copper-Molybdenum-Sulfur Interaction

In the presence of ruminal H^+ ions, dietary S is reduced to sulfide, which then reacts with Mo (Mason, 1986) to form different thiomolybdates (mono, di, tri, tetrathiomolybdates) according to the following reactions:

$$MoO_4^{2-} + H^+ + HS^- \leftrightarrow H_2O + MoO_3S^{2-} \text{ (monothiomolybdate)}$$


$$MoO_3S^{2-} + H^+ + HS^- \leftrightarrow H_2O + MoO_2S_2^{2-} \text{ (dithiomolybdate)}$$


$$MoO_2S_2^{2-} + H^+ + HS^- \leftrightarrow H_2O + MoOS_3^{2-} \text{ (trithiomolybdate)}$$


$$MoOS_3^{2-} + H^+ + HS^- \leftrightarrow H_2O + MoS_4^{2-} \text{ (tetrathiomolybdate)}$$

In the gastrointestinal tract, thiomolybdates have been shown to bind Cu preventing its absorption, while increasing the Cu fraction associated with the solid phase of the rumen content at expense of a reduction in the fluid phase. Thiomolybdates associated with solid rumen digesta (bacteria, protozoa, and indigested feed particles) form insoluble complexes that do not release Cu even under acidic conditions like the abomasal environment (Allen and Gawthorne, 1987). In addition, absorbed thiomolybdates have also been shown to cause systemic effects on Cu metabolism including increased biliary excretion of Cu from liver stores, strong binding of Cu to plasma albumin resulting in reduced availability for biochemical processes, and inhibition of Cu dependent metalloenzymes such as ceruloplasmin, diamine oxidase, cytochrome oxidase, ascorbate oxidase and tyrosine oxidase. (Mason, 1986; Suttle, 1991; Spears, 2003). According to Spears (2003), when rumen sulfide concentrations are low, Mo has little effect on the formation of thiomolybdates. However, Cu bioavailability is deeply reduced (up to 70%) when Mo levels are not modified but sulfide concentrations are increased (Suttle, 1975).

Copper-Sulfur Interaction

In addition to its role in the Cu-Mo-S interaction, organic or inorganic S can also reduce Cu bioavailability *per-se* (Spears, 2003). Van Ryssen et al. (1998) found a

55% reduction in hepatic Cu when sheep were fed high levels of S (2 g/kg DM). According to the authors, this reduction was due to formation of CuS in the digestive tract, since the diet was very low in Mo. Similar results were previously observed by Suttle (1974) who found a 39-56% reduction in Cu bioavailability when S was provided to ewes as methionine or as $NaSO_4$ under low Mo dietary levels, possibly through the formation of insoluble CuS at sites beyond the rumen. However, Jouany (1991) postulates that the formation of insoluble CuS and Cu_2S in the rumen is exacerbated by the digestion of insoluble proteins by protozoa, with the consequent increase in available S. Despite the effect of dietary S previously mentioned, other S sources have been also responsible for decreasing Cu bioavailability in ruminants. Molasses, a by-product from the sugarcane and beet industry, is a source of dietary sugars feed to dairy cows (NRC, 2001). Benefits of adding molasses to diets include increasing palatability, acting as a binder, and reducing dust in fine-particle feeds (Morales et al., 1989). However, due to its high content of S, the liberal use of molasses can result in dietary S levels considerably in excess of requirements (Zinn et al., 1997). Arthington and Pate (2002) evidenced a decrease in liver Cu at 29, 56 or 84 d after feeding heifers with a molasses based supplement. According to the authors, this observation was the result of high concentrations of S naturally found in molasses.

In a review of Cu antagonists in cattle, Arthington (2003) describes other sources of S implicated in the Cu-Mo-S and Cu-S interactions, included fertilizers, high S water, and S containing supplements. Cows grazing bahiagrass pastures

fertilized with ammonium sulfate showed lower liver Cu concentrations compared with cows on non fertilized pastures, or fertilized with ammonium nitrate (Arthington, 2003). A previous study from Spears et al. (1985) showed that gypsum fertilization (132 kg S/ha) increased S from 0.33 to 0.40% and from 0.29 to 0.37% of DM in tall fescue grass and in orchadgrass, respectively. However, feeding those pastures to steers resulted in no changes in Cu bioavailability, probably due to the high S content of the non fertilized pastures. For this reason Arthington et al. (2002) suggest that the choice of fertilizer source can be critical in areas where grazing cattle may be prone to Cu deficiency.

Sulfur levels in drinking water can also be detrimental for Cu bioavailability. Cammack et al. (2010) reported a decrease in plasma and hepatic Cu of yearling steers provided with high-S water (3,651 mg of SO_4/L) compared with those receiving low-S water (566 mg of SO_4/L). Similarly, a decrease in hepatic Cu of growing steers was found by Wright and Patterson (2006) when S content in the drinking water was increased from 404 to 4654 mg of SO_4/L. While the mentioned S concentrations are in excess of that commonly found in water for animals, high S-containing water has been reported in USA and Canada (Paterson et al., 1999; Haydock, 2003).

Copper-Iron Interaction

Ruminants consuming forage-based diets are often exposed to high levels of Fe through water, forage, and unusually high amounts of soil ingestion (Suttle et al.,

1975; Mullis et al., 2003). Supplementing 800 mg of Fe/kg of DM as FeO or $FeSO_4$, decreased Cu absorption from 0.06 to 0.04 in sheep (Suttle and Peter, 1985). In agreement, a previous study of Humphries et al. (1983) found a rapid decrease in liver and plasma Cu concentrations, activities of erythrocyte superoxide dismutase and plasma ceruloplasmin of young heifers receiving 800 mg of Fe/kg of DM. However, according to Suttle (2010) the role of Fe on Cu absorption is partially dependent of S. Indeed, Suttle et al. (1984) suggest that the formation of FeS in the rumen is a critical step for Fe to antagonize Cu absorption. An alternative explanation is provided by Arredondo et al. (2003) who demonstrate that excess Fe can compete with Cu for its absorption at intestinal level, by saturating the DMT-1 Cu transporter.

2.5.2. Role of copper on rumen fermentation

In a production study conducted on beef steers (Engle and Spears, 2000b), the addition of 20 or 40 mg of Cu/kg of DM decreased animal performance, compared with animals receiving a basal diet containing 10.2 mg of Cu/kg of DM, suggesting that high dietary Cu may inhibit ruminal fermentation. Previously, Essig et al. (1972) found a decrease in post feeding total VFA concentration and VFA molar proportions of yearling steers receiving a high dose of supplemented Cu (57.3 mg/kg of DM), but average daily gain, feed efficiency, and carcass yield and quality grade were not affected. In agreement, Slyter and Wolin (1967) found an *in vitro* depression in rumen fermentation of concentrates following the addition of high

doses of $CuSO_4$. Similarly, Piva et al. (1986) found a reduction in propionate molar proportion when high doses of Cu were added to *in vitro* rumen incubations. A dose-response study conducted by Forsberg (1978) determined that 21 µg of Cu/mL incubation fluid was required to obtain a 50% inhibition of gas production. However, a large disparity was obtained regarding the susceptibility of the different bacterial populations to Cu. While the growth of *Bacteroides succinogenes, Ruminococcus albus* and *Butyrivibrio fibrisolvens* was inhibited by 10, 20 and 30 µg Cu/mL of incubation fluid, respectively, higher concentrations were required to inhibit *Megasphaera elsdenii*, *Selenomonas ruminantium*, and *Streptococcus bovis* (100, 100 and 250 µg Cu/mL, respectively).

Opposite results were reported by Zhang et al. (2007) who found a reduction in rumen pH and an increase in total VFA concentrations when Cashmere wether goats received supplementary Cu in the diet. According to the authors, an increase in NDF digestion may be responsible for these findings. Conversely, later studies conducted by Zhang et al. (2008; 2009) found that NDF digestion was unchanged or maximized by the addition of 10 mg of Cu/kg of DM, but depressed when of 30 mg of Cu/kg of DM were added. In addition, no differences were obtained for rumen pH, IVDMD and VFA molar proportions when *in vitro* studies were conducted using rumen fluid donors receiving 0, 10 or 20 mg of Cu/kg of DM (Engle and Spears, 2000a). Based on previous observations showing that 20 or 40 mg of Cu/kg of DM increased unsaturated fatty acids in adipose tissue of steers (Engle and Spears, 2000a), a possible role of Cu as an inhibitor of ruminal lipids biohydrogenation has

been suggested. (Engle, 2011) However, no studies have been conducted to assess the validity of this hypothesis.

2.5.3. Copper availability from organic and inorganic sources

The effectiveness of organic sources of Cu to promote animal benefits has been a subject of several controversies. Nockels et al. (1993) reported a higher retention of Cu in steers supplemented with Cu-lysine compared with supplementation with $CuSO_4$. In another study (Ahola et al., 2004), the use of organic Cu (as Cu proteinate) increased hepatic retention of Cu in multiparous beef cows, compared with inorganic Cu. However, no benefits on cow and calf performance were obtained. Conversely, Datta et al. (2007) found an increase in body weight gain in goat kids supplemented with organic Cu, compared with inorganic Cu. In a recent meta-analysis (Rabiee et al., 2010) assessing the benefits of organic trace minerals, only marginal improvements in milk production, milk fat and milk protein were found. In opposition, organic trace minerals did not affect somatic cell count, interval from calving to first service, and 21-d pregnancy rate. Similarly, no differences in 60-d pregnancy rate, health or performance were found by Muehlenbein et al. (2001) in 2-year old cows receiving Cu as $CuSO_4$ or as amino acid complex. In addition, Olson et al. (1999) found a decrease in pregnancy rates of primiparous cows receiving organic and inorganic minerals (Cu, Co, Mn and Zn) compared with non-supplemented cows. According to the authors, excessive supplementation beyond requirements reduced reproductive performance. In a previous study conducted on steers (Ward et al.,

1993), growth rate was higher when animals received $CuSO_4$ than when Cu-lysine was provided during the initial 21 d, but no differences were obtained after 98 d. Other parameters, including feed efficiency, feed intake, humoral and cellular immune response and ceruloplasmin activity, were not affected by Cu source (Ward et al., 1993). Organic sources of Cu have been seriously criticized by Underwood and Suttle (2001) who consider that technologies for protection against rumen antagonisms are extravagant and provide no additional benefits than conventional $CuSO_4$. In addition the authors questioned the scientific legitimacy of some *in vivo* studies where no proper covariance analyses were conducted to account for initial differences between groups of animals (Underwood and Suttle, 2001). According to Suttle (2010) commercially driven pursuit of trivial advantages over cheap and effective inorganic sources of Cu should cease, and attention should focused on predicting when supplementation is needed and to the usual problem of over, rather than under-provision of Cu and its environmental impact.

In conclusion, Cu, Zn and Mn are required to maintain health and production status of livestock, but their functions at the gastrointestinal tract of ruminants are not totally elucidated. Like many other minerals, Cu, Zn and Mn have the ability to interact with organic compounds of the diet, macro minerals and micro minerals, usually resulting in decreased availability for the host.

Different technologies, including proteinates, amino acid chelates, amino acid complex, and polysaccharide complex are currently available for mineral protection.

However, these technologies appear to be more effective in monogastrics than in ruminants.

Mechanisms to guarantee optimal levels of ruminally available minerals, and to optimize mineral supply to the lower gastrointestinal tract without compromising postruminal absorption require further research.

3. RUMINAL SULFUR AND HYDROGEN SULFIDE TOXICITY`

3.1. Introduction

Sulfur is one of the most abundant elements in nature and constitutes 0.15% of the body weight of mammalians with individual tissues varying from 0.5 to 2% of their weight as S (NRC, 2001; Suttle, 2010). Despite its abundance, a shortage of sulfur-containing amino acids is a worldwide problem in animal nutrition. Sulfur is a structural component of several compounds including methionine, cystine, cysteine, homocysteine, taurine, and S-adenosyl methyltransferase (McDowell, 1992). In addition to amino acids, S is present in the chondroitin sulfate of cartilage and in the B-vitamins thiamin and biotin, and is part of the disulfide bonds responsible for maintaining the tertiary structures of protein molecules (Ammerman, 1995). Realistically, S is an essential nutrient for only plants and microbes, because they have the ability to synthesize sulfur amino acids from inorganic sources. In ruminants, dietary S either as S-containing amino acids or inorganic S is reduced to sulfide by rumen microorganisms and then incorporated

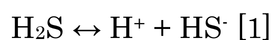
into microbial protein or absorbed and oxidized to sulfate in the liver (Kandylis, 1984). For this reason, dietary requirements of S are primarily determined by its essentiality for microbial protein synthesis and are usually expressed as a fraction of the dietary N. Bouchard and Conrad (1973) suggested that an optimal N:S ratio of 10 to 12:1 for efficient utilization of nonprotein N. A 14:1 ratio has been adopted by the ARC (1980). Currently, the requirement of S to sustain growth and production in dairy cows is set at 0.15 to 0.2% of dietary DM (NRC, 2001; CSIRO, 2007), with adequate S availability obtained by supplementing inorganic sources of S (ammonium bisulfate, ammonium sulfate, calcium sulfate, sodium bisulfate and sodium sulfate), or organic sources (methionine and methionine hydroxyl analogs), but probably not with cysteine or elemental S (Ammerman, 1995). However, the margin between desirable and toxic concentration of S in ruminant diets is surprisingly small, with negative effects reported at 0.3 to 0.4 % S in DM for dairy cattle (Kandylis, 1984) and 0.25% S in feedlot steers (Loneragan et al., 2001). In addition to dietary S, other sources of S have been identified as contributors to high S intake, including drinking water (Hamlen et al., 1993; Haydock, 2003) and S-rich molasses (Mella et al., 1976; Niles et al., 2000).

3.2. Mechanism of sulfur toxicity

Excessive S in the diet can be detrimental for absorption of other elements like Cu, due to the formation of unavailable mineral complexes (Jouany, 1991; Arthington, 2003; Spears, 2003). According to several authors, the main mechanism responsible for S toxicity in ruminants is the ruminal formation of hydrogen sulfide (H_2S) and its hydrosulfide anion (HS^-) form (Bird, 1972; Beauchamp Jr. et al., 1984; Suttle, 2010). Although there appear to be no published studies that have compared inorganic S sources on kinetics of rumen H_2S release, Dewhurst et al. (2007) found that H_2S production was greater and more rapid when cows received cysteine vs. methionine. This was presumably due to formation of compounds such as methanethiol and dimethyl sulfide with the latter but not with the former (Zikakis and Salsbury, 1969). Reduction of dietary sulfate in the rumen is mediated by microbes. Sulfate-reducing bacteria are classified as assimilatory or dissimilatory. Assimilatory sulfate reducers, including *Bacteroides, Butyrivibrio* and *Lachnospira*, incorporate the reduced sulfate into S-containing aminoacids, while generating limited amounts of sulfide for metabolic requirements. Conversely, dissimilatory sulfate reducers are strict anaerobes that use sulfate as a terminal electron acceptor to derive energy for growth and metabolism. This reaction is coupled to the oxidation of lactate, formate or pyruvate, and the organisms usually produce more H_2S than necessary for bacterial growth. *Desulfobrivio* and *Desulphotomaculum* are the most representative of the dissimilatory sulfate reducers (Cummings et al., 1995a; Hobson and Stewart, 1997). The generation of large quantities of H_2S in the rumen can

cause severe distress of the nervous system, and is frequently associated with polioencephalomalacia (PEM), a condition characterized by cerebrocortical necrosis (Gould et al., 1997; Cammack et al., 2010). During the course of the disease, large quantities of H_2S are thought to be absorbed by the lungs due to inhalation of the eructed gases (Dougherty et al., 1962; Bulgin et al., 1996). Because this route of absorption circumvents potential hepatic detoxification mechanisms, delivery of H_2S to sensitive tissues could be facilitated and toxicity enhanced (Cummings et al., 1995a), causing pulmonary edema (Beauchamp Jr. et al., 1984) and PEM (Gould, 2000). Until recently, H_2S toxicity was related to its capacity for direct inhibition of cytochrome oxidase, an enzyme critical for mitochondrial respiration in a similar manner to cyanide (Dorman et al., 2002), with high oxygen demand tissues (e.g., nervous and cardiac tissue) being the most sensitive to disruption of oxidative metabolism by H_2S (Ammann, 1986). However, Truong et al. (2006) proposed a complementary mechanism that involves generation of reactive sulfur species, with a depletion of glutathione (GSH) and an increase in the formation of reactive oxygen species.

In aqueous solutions, H_2S exhibits two acid dissociation constants. The first dissociation occurs with ionization of a single proton and yields a hydrosulfide anion. The second dissociation occurs from the resultant hydrosulfide anion to give the sulfide ion, according to the following equations:



$$H_2S \leftrightarrow H^+ + HS^- \quad [1]$$

$$HS^- \leftrightarrow H^+ + S^{2-} \quad [2]$$

The pK_a, values for dissociation equations [1] and [2] are 7.04 and 11.96, respectively (Beauchamp Jr. et al., 1984). For that reason, ruminal pH is a critical factor in determining the occurrence of different chemical species. While the proportion of H_2S:HS^- is 46.8:50.4 at a ruminal pH of 6.8, this ratio increases to 97.2:2.8 when ruminal pH decreases to 5.2, indicating a role of dietary concentrates in exacerbating ruminal production of H_2S (Kung et al., 2000).

The course of S intoxication appears variable and species related. In accidentally S-intoxicated steers, Haydock (2003) reported a period of 48 h as necessary for the onset of clinical signs. Similarly, Radostits et al. (2000) noted a period of 24 to 48 h for deaths to occur in feedlot steers. Hamlen et al. (1993) observed a 3-d delay following S intoxication in Holstein cows for clinical signs to occur. In an accidental S intoxication in dairy cows, an interval of 3 to 10 d was necessary for deaths to occur (Gunn et al., 1987). However, in an experimentally induced PEM study conducted in steers, Cummings et al. (1995b) noticed that neurological signs appeared after an adaptation period of 10-14 d. Similarly, a 3-week delay in the presentation of clinical signs was noticed by Gooneratne et al. (1989) when sulfur induced PEM was experimentally reproduced in sheep. A 3 to 7-week delay was reported by Rousseaux et al. (1991) and by Olkowski et al. (1992) in lambs and sheep receiving high levels of dietary S for PEM to occur. As a possible explanation, Cummings et al. (1995a) indicated that rumen fluid from steers fed a diet high in S had a greater capacity for reduction of sulfate to sulfide, probably related to microbial adaptation to high S conditions, but no studies have been

conducted regarding the dynamics of S-assimilatory and S-dissimilatory rumen microbial populations following the addition of S to the diet.

3.3. Prevention and treatment of polioencephalomalacia

Several studies conducted on animals affected by PEM showed a decrease in thiamine concentration in body tissues (Rammell and Hill, 1986; Gooneratne et al., 1989), or an increase in levels of thiamine-destroying thiaminases at the gastrointestinal tract (Edwin and Jackman, 1982; Ramos et al., 2005). For this reason, thiamine supplementation has been proposed as a method to prevent and treat PEM in ruminants (Olkowski et al., 1992; McDowell, 2000). Some cases of intravenous thiamine hydrochloride treatment at the onset of PEM resulted in animal recovery, however other studies showed no relationship between tissue and ruminal fluid thiamine concentrations and PEM (Gould, 1998). Furthermore, episodes of PEM have been observed in weaned calves showing excessive S intake and high ruminal H_2S concentrations, without evidence of thiamine deficiency (Loneragan et al., 1998a). In addition, some studies showed a lack of protective effect of thiamine in experimentally induced S intoxication (Ward and Patterson, 2004; Neville et al., 2010). For this reason, Galyean and Rivera (2003) concluded that the role of thiamine in PEM is not definitive.

Based on the ability of Mo to bind sulfur, Kung et al. (2000) explored the use of Mo to reduce H_2S production. Addition of 10 and 25 ppm Mo to *in vitro* incubations of rumen fluid from a steer with a high S diet resulted in a reduction in H_2S in

headspace gas, but no differences with the control were obtained when Mo was added at 1 ppm. Similarly, a reduction in H_2S from *in vitro* incubations was found by Ruiz-Moreno et al. (2010) following the addition of sulfate salts of Zn, Mn, and Cu compared with polysaccharide protected Zn, Mn, and Cu, probably due to a higher formation of S complexes and a reduction in S availability. In agreement, excess of Fe and Zn resulted in lower H_2S production in fecal slurry from human patients (Mitsui et al., 2003). However, utilization of antagonist minerals must be carefully evaluated because a dramatic decrease in liver stores of Cu was found by Loneragan et al. (1998b) following supplementation of sodium molybdate to achieve a H_2S reduction in the rumen gas cap of cattle.

Based on observations from Herszage and dos Santos Afonso (2003), Kelzer et al. (2010b) proposed the utilization of MnO for H_2S reduction, but no beneficial effects were obtained either *in vitro* or *in vivo* (Kelzer et al., 2010a; b). Conversely, a dramatic decrease in H_2S production was observed *in vitro* by Kung et al. (1998; 2000) following the addition of 1, 10 or 25 ppm of 9, 10 anthraquinone, an active component of aloe (*Aloe succtrina*) and senna (*Senna alexandria*) oils. While no toxic effects have been found following supplementation of 66 ppm of 9, 10 anthraquinone to the diet of sheep (Kung Jr. et al., 2003), the associated reduction in methane production and increase in rumen hydrogen suggest a loss of energy for the host animal (Calsamiglia et al., 2007).

In an attempt to find rumen fermentation modifiers with a capability to reduce H_2S, the effect of several ionophores was examined by Kung et al. (2000). *In vitro*

rumen fluid incubations containing bacitracin (1.25 ppm), oxytetracycline (1.25 ppm), chlortetracycline (5 ppm), lasalocid (5 ppm), bambermycin (0.3 ppm), monensin (5 ppm), avoparcin (5 ppm) and tylosin (1.5 ppm) were conducted. No effect on H_2S release was found following the addition of bacitracin, lasalocid, bambermycin, avoparcin and tylosin. However, a 72 and 55% reduction in H_2S was detected following addition of chlortetracycline and oxytetracycline, respectively. According to the authors, this difference can be related to the activity of the different antibiotics. While chlortetracycline and oxytetracycline are classified as broad-spectrum antibiotics, with activity against Gram-negative and Gram-positive bacteria, the others are known to have activity against Gram-positive bacteria only. Interestingly, a 50% increase in H_2S production was obtained when monensin was added to the incubation media. The authors hypothesized that indirect inhibition of methanogens by monensin may have decreased competition between methanogens and sulfate-reducing bacteria, but the specific reason for a stimulatory effect of monensin on sulfide production is not identified. Conversely, no effect of antibiotics was shown by Quinn et al. (2009) when lasalocid (5 ppm), monensin (5 ppm), laidlomycin (1.65 ppm), chlortetracycline (5 ppm) and tylosin (1.25 ppm) were added alone or in combination to rumen fluid incubations. According to the authors, differences in fermentation substrate among experiments may be partially responsible for the controversy of these findings. In addition, while in the latter study S was added at 0.42% of DM, a final concentration of 1.09 % DM was added in the former study, suggesting that a greater S concentration may induce some

interactions that are not present when S concentrations are closer to physiological conditions (Quinn et al., 2009).

Besides its effects in ruminants, H_2S has been associated with the onset of ulcerative colitis, a condition predisposing to colorectal cancer in humans (Furne et al., 2000; Huycke and Gaskins, 2004; Rowan et al., 2009). In addition, excessive production of H_2S has been related to the unpleasant odor of flatulence in humans and dogs (Collins et al., 2001). In the colon of individuals harboring sulfate reducing bacteria, production of H_2S appears to be largely dependent on metabolic activity of these microorganisms that utilize sulfate as an electron acceptor during dissimilation of organic compounds such as acetate, propionate, lactate, butyrate, succinate, ethanol, pyruvate, some amino acids (Gibson et al., 1993).

In an attempt to decrease H_2S production in humans, Suarez et al. (1998) proposed the use of bismuth subsalicylate (BSS), a compound that avidly binds sulfide, presumably in the form of insoluble bismuth sulfide (Bi_2S_3), according to the following reaction:

$$2\ Bi^{3+} + 3\ H_2S \rightarrow Bi_2S_3 + 6\ H^+$$

The authors conducted a series of *in vitro* and *in vivo* studies with rats and human patients. Compared with controls, addition of BSS at 2.6 μmol/mL of rat and human fecal homogenates reduced H_2S by 99.93, 99.97, and 99.99% at 2, 4, and 24 h, respectively. In the same study, patients receiving 400 mg BSS *per os* exhibited a 96% decrease in H_2S release. Similarly, a drastic reduction in H_2S production was

found by Furne et al. (2000) following the administration of BSS (0.68% of dietary DM) to rats fed a poorly absorbable source of S. In a later study, addition of BSS at 0.1, 0.5 and 1.0 mmol/L resulted in a >90% reduction in H_2S from batch cultures containing fecal slurry from healthy humans (Mitsui et al., 2003). Besides its ability to decrease H_2S by a binding mechanism, BSS has antimicrobial properties (Sox and Olson, 1989) and has been extensively used in the treatment of traveler's diarrhea (Ericsson, 2005; Dupont, 2008). Cornick et al. (1990) found a high variability in the 90% minimal inhibitory concentration of BSS depending on the bacterial species. While 90% reduction of *Clostridium difficile* was achieved by 128 μg of BSS/mL of incubation media, a similar reduction in *Escherichia coli* and *Staphylococcus aureus* was achieved with 4,096 μg/mL, and 6,144 μg/mL were required to inhibit 90% of *Pseudomonas aeruginosa*. Conversely, Gorbach et al. (1990) found no changes in total microbial counts or in counts of individual groups such as enteric bacteria, *Pseudomonas*, *Staphylococcus*, *Bcteroides*, or *Clostridium difficile* in stools of humans receiving up to 453 g of BSS during a period of 48 h.

At the present time, BSS is available for short-term use as an anti-diarrheal for humans (Dupont, 2008), calves (Roussel Jr. and Brumbaugh, 1991), foals (Becht and Byars, 1986), horses (Clark et al., 1996) dogs and cats (Papich et al., 1987). In addition to the potential benefits of BSS, bismuth preparations are generally considered to be safe due to their low absorption. However, accidental cases of toxicity have been reported in humans following the ingestion of large doses of BSS during prolonged periods of time, mainly due to unsupervised and indiscriminate

use (Lambert, 1991; Gordon et al., 1995). In humans, clinical signs of Bi intoxication typically include a gradual subacute progressive encephalopathy, associated with confusion, memory difficulty and in some cases delirium (Gordon et al., 1995). Bismuth neurotoxicity was successfully induced in mice following intraperitoneal injection of Bi when concentrations in brain tissue reached 8,000 ng/g (Bierer, 1990). However, no toxicity signs were observed when mice received 60-fold the maximum recommended dose of BSS during 91 d (Ross et al., 1988). According to Gavey et al. (1989) only 0.2% of ingested bismuth is absorbed in humans, presumably because of the insolubility of bismuth salts in the stomach and intestine (Bierer, 1990). However this determination is questionable because it did not account for temporary storage of bismuth in body compartments or for possible enterohepatic circulation (Lambert, 1991). At present, no studies are available regarding the toxicity of BSS in adult ruminants, however special caution is advised for its use in cats, due to their inability to rapidly metabolize and excrete salicylates (Perkins et al., 1996; Richardson, 2000). While no known research has been conducted examining the effects of BSS on ruminal fermentation, its utilization seems a promising alternative for the mitigation of rumen H_2S production.

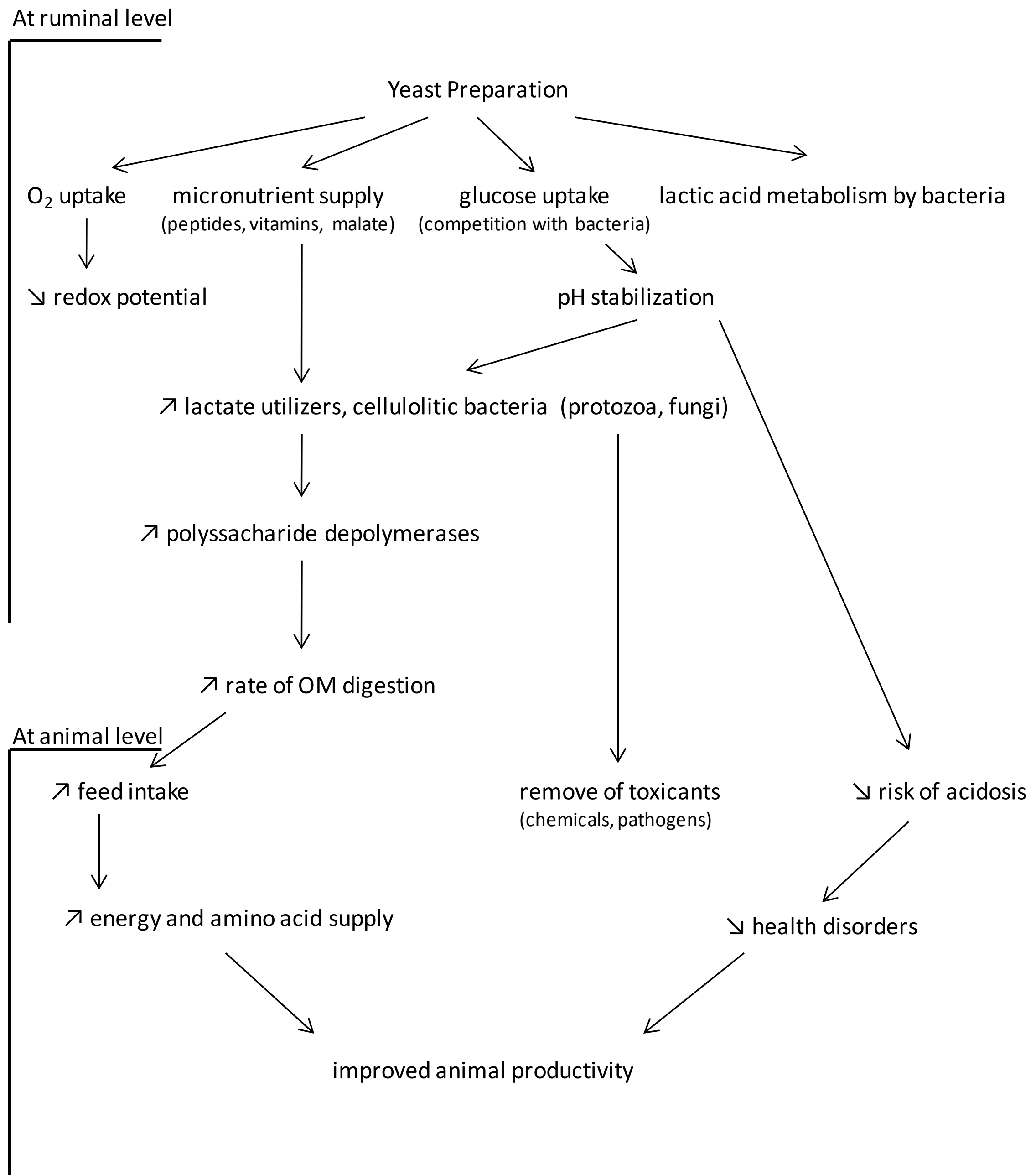


Figure 1.1: Proposed model to describe the action of yeasts in ruminants (Jouany and Morgavi, 2007)

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Chapter 2 : Effects of *Saccharomyces cerevisiae* on fermentation by ruminal microbes maintained in dual flow continuous culture fermenters

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Abstract. Effects of *Saccharomyces cerevisiae* (SC) on rumen fermentation were evaluated using a dual flow continuous culture system. Eight fermenters were inoculated with ruminal fluid from a dairy cow in early lactation on d 1 of a 10-d experimental period. Fermenters were provided with 75 g of DM/d of a pelleted diet formulated for a high lactating dairy cow (40 kg milk/d, 3.8% fat, 3.7% protein). Two levels of active dry yeast (Levucell, SC20, Lallemand) at 0 or 2 mg/fermenter/day (NY and YS, respectively) were infused twice daily at 0900 and 2100 h to fermenters in a completely randomized arrangement of treatments. The latter concentration would be equivalent to supplementing 0.5 g/d of SC20 to a dairy cow. Apparent and true organic matter degradability were not affected ($P > 0.05$) by SC averaging 55.6

vs. 56.0 and 65.6 vs. 66.5 % for NY and YS, respectively. No differences were obtained ($P > 0.05$) in neutral detergent fiber (NDF) and acid detergent fiber (ADF) digestibility (51.1 vs. 49.4% and 50.3 vs. 48.1% for NY and YS, respectively). Total volatile fatty acid (VFA) concentrations were not affected ($P > 0.05$) by treatments (92.8 and 93.1 m*M* for NY and YS, respectively). Addition of SC did not affect VFA molar proportions or estimated CH_4 production but resulted in a trend ($P = 0.1$) for a lower A:P ratio at 2.49 vs. 2.10 for NY and YS, respectively. Addition of SC decreased ($P < 0.05$) ammonia nitrogen (NH_3-N) concentration and NH_3-N daily flow (6.28 vs. 3.85 mg/100 mL and 0.16 vs. 0.10 g/d for NY and YS, respectively), without affecting ($P > 0.05$) crude protein degradation and efficiency of microbial protein synthesis (58.0 vs. 50.9 % and 29.1 vs. 25.8 g of N/kg organic matter truly digested for NY and YS, respectively). Mean and minimum pH of fermenters did not differ ($P > 0.05$) between treatments but a trend ($P < 0.1$) for a lower maximum pH was obtained at 5.78 vs. 5.71 for NY and YS, respectively. Time spent below pH 5.6, between 5.6 to 5.8, or above 5.8 was not influenced by treatments ($P > 0.05$). In conclusion, a low dose of active dry yeast decreased NH_3-N concentration and daily flow, without affecting any other of the *in vitro* rumen fermentation characteristics measured in this study.

Keywords: *Saccharomyces cerevisiae*, rumen, continuous culture fermenters

1. INTRODUCTION

Several direct feed microbials (DFM), including *Lactobacillus* spp., *Bifidobacterium* spp., *Lactococcus* spp., and *Saccharomyces cerevisiae* (SC), are currently available in the animal nutrition market (Oetzel et al., 2007). Of all of the DFM, preparations containing live SC are the most commonly marketed for ruminants (Chaucheyras-Durand and Durand, 2010). Commercially available formulations containing SC include active dry yeast that contains live SC cells either alone or with a small amount of carrier, while others contain the dehydrated microorganism and the medium it was grown on (Lynch and Martin, 2002). Benefits of SC as a probiotic for ruminants have been reported throughout the years (Williams et al., 1991; Oeztuerk and Sagmanligil, 2009) including effects on rumen and lower gastrointestinal tract (Bach et al., 2007; Stella et al., 2007), immune response (Comitini et al., 2005; Galvao et al., 2005), and production characteristics of beef and dairy cattle (Plata et al., 1994; Wang et al., 2001; Stella et al., 2007). Effects of yeast in the rumen seem to be mediated by oxygen scavenging (Newbold, 2007), reduction in lactate concentration (Callaway and Martin, 1997; Marden et al., 2008), pH stabilization, decreases in the risk of subclinical rumen acidosis (Bach et al., 2007; Thrune et al., 2009), changes in volatile fatty acids (VFA) metabolism (Miller-Webster et al., 2002), and improved fiber digestion (Guedes et al., 2008). In addition, controversial results on methane (CH_4) production have been observed (Martin et al., 1989; Chaucheyras et al., 1995b). However a complete understanding

of yeast mechanism in ruminants remains unclear (Moallem et al., 2009). Many of the cited effects can be attributed to SC *per-se*, while others seem to be related to their interaction with protozoa populations (Brossard et al., 2006; Fonty and Chaucheyras-Durand, 2006). In addition, effects of SC may depend on dietary characteristics (Chaucheyras-Durand et al., 2008). Dual flow continuous culture fermenters are used as a model for studying rumen metabolism, but is usually lacking in protozoa when high liquid dilution rates are applied (Mansfield et al., 1995; Stern et al., 1997; Ziemer et al., 2000). The objective of the present experiment was to evaluate the effect of active dry yeast on rumen bacterial fermentation using dual flow continuous culture fermenters receiving a diet formulated for a dairy cow in early lactation.

2. MATERIALS AND METHODS

2.1. Experimental diet

A diet was formulated for a lactating dairy cow based on NRC (2001) recommendations to meet the requirements of a 650 kg Holstein cow producing 40 kg of milk with 3.8% fat and 3.7% protein. Ingredient and chemical composition of the diet is shown in Table 2.1 and Table 2.2, respectively. All ingredients of the basal diet were ground through a 2-mm screen (Thomas - Willey laboratory mill

Model 4, PA, USA) and pelleted with a CL-5 California Pellet mill (California Pellet Mill Co., Crawfordsville, IN) to a final dimension of 6 mm in diameter x 12 mm long.

2.2. Continuous culture operation

Eight dual flow continuous culture fermenters, as described by Hannah et al. (1986) with a modified pH control and measuring system, were randomly assigned to either 0 or 2 mg of *S. cerevisiae* (strain CNCM-1077, Levucell SC20® SC, Lallemand Animal Nutrition, 2 x 10^{10} CFU/g) /fermenter/d during a 10-d experiment. The latter concentration would be equivalent to supplementing 0.5 g/d to a lactating dairy cow consuming 18.75 kg DM per day. Fermenters were provided with 75 g of DM per day of the pelleted diet (Table 2.2), divided in eight equal portions by an automated feeding system. Each portion was provided during a 90-min period beginning at 0300, 0600, 0900, 1200, 1800, 2100 and 2400 h daily. One hundred mg of *S. cerevisiae* were solubilized in 100 mL saline solution (0.9% w/v), and one mL of the solution was infused in the fermenters twice daily at 0900 and 2100 (Yeast supplemented group, YS). Fermenters that were used as the control group received 1 mL of saline solution (No yeast group, NY) at the same time. Infusions were delivered into the fermenters below the overflow port at a rate of 1 mL/min with a screw-driven constant-infusion pump (Harvard Apparatus Co., Holliston, MA).

Liquid flow rate of fermenters was set at 10%/h by regulating artificial saliva (pH = 8.25) input while solids dilution rate was set at 5.5%/h by regulating liquid output through filters. Individual pH was recorded every 5 min by an electronic data

acquisition system (Daisy Lab®) and was maintained between 5.5 and 6.5 by automated addition of either 5N NaOH or 3N HCl. Anaerobic conditions were maintained by continuous infusion of N_2 at a rate of 40 mL/min throughout the experiment. Fermenter temperature was maintained at 39.1 ± 0.1°C by an electrical heater.

2.3. Sample collection and analytical procedures

After a 7-d adaptation period, fermenter effluents were maintained at 1°C in a water bath to retard microbial and enzymatic activities. During three consecutive days, fermenter effluents were homogenized using a PT10/3S homogenizer (Kinematica GmbH, Bohemia, NY), and three separate 500 mL aliquots were removed daily and composited by fermenter. Composite effluent samples were analyzed for total-N, ammonia-N (NH_3-N) and VFA. Freeze-dried composite samples were analyzed for DM, OM, NDF, AFD, ash, and purines. Dry matter content of diets, outflows and microbial pellets was determined by drying in an oven at 105°C for 24 h. Ash was determined by weight difference after 24 h combustion at 550°C (AOAC, 1984). At the end of each experimental period, contents from each fermenter were strained through two layers of cheesecloth, centrifuged at 1,000 x *g* for 10 min to remove feed particles and the supernatant was centrifuged at 20,000 x *g* for 20 min to isolate bacteria. Bacterial pellets were resuspended in distilled water, frozen and lyophilized. Purine concentrations were determined by the method of Zinn and Owens (1986). Purine content of effluent and bacteria was used to partition flow of

effluent N into microbial and dietary N. Total N in the effluent, bacteria, and diet was determined (AOAC, 1984). Ammonia-N was determined by steam distillation (Bremner and Keeney, 1965) using a Kjeltech 2300 Analyzer Unit (Tecator, Herdon, VA). Sequential detergent fiber analyses (Van Soest et al., 1991) were used to determine NDF and ADF concentrations of diet and effluents.

Effluent VFA concentrations were determined by capillary GC analysis. Incubation fluid was solvent-extracted using ethyl acetate (3:7 ratio) during 10 minutes under continuous vortex. Samples were centrifuged at 5,000 *g* for 5 min and supernatant was stored at -20 °C until analyzed. Analysis was performed using a Hewlett-Packard 5890 GC equipped with a Stabilwax-DA capillary column (30 m x 0.25 mm i.d., 0.25 μm film thickness, Restek, CA). Chromatographic conditions were: helium (1.7 mL/min), initial oven temperature, 110°C, held for 2.1 min; ramped at 25°C/min to 200°C, held for 1.5 min; injector temperature: 200°C, flame ionization detector temperature: 220°C; split injection (split ratio: 1/10); injection volume: 1 μL. Ethyl butyrate was included as an internal standard. Stoichiometric methane production was calculated as described by Hegarty and Nolan (2007).

2.4. Calculations and Statistical analyses

Data from fermenter effluent were analyzed as a completely randomized design using one way analysis of variance. Differences were considered significant at $P \leq 0.05$ and tendencies discussed at $P \leq 0.1$. Data for fermentation pH that was obtained on 3 consecutive days were analyzed as repeated measures with an

autoregressive order 1 structure of covariance, based on minimum values of AIC (Akaike's Information Criterion). Lowest and highest hourly recorded pH were used for minimum and maximum pH comparison between treatments. Time (min) spent below pH 5.6, between pH 5.6 to 5.8 and above pH 5.8 was calculated using the trapezoidal integration method. Comparisons between treatments were conducted by one way analysis of variance. Data processing was conducted using the statistical software R (R Development Core Team, 2005). Unless noted otherwise, results are reported as least squares means from 4 observations/treatment.

3. RESULTS AND DISCUSSION

Effects of SC on OM and fiber digestibility are presented in Table 2.3. Apparent and true OM digestion did not differ ($P > 0.05$) between treatments. These results are in agreement with previous results (Moloney and Drennan, 1994; Enjalbert et al., 1999; Moallem et al., 2009). However, others have reported a decrease in *in sacco* dry matter degradability in small ruminants receiving live yeast (Flachowsky et al., 1992) and others have shown a positive effect of live yeast or yeast culture on OM digestion in different species including cattle, goat and sheep (Wiedmeier et al., 1987; Paryad and Rashidi, 2009). Improvements in OM digestion appear to be related to the ability of SC to increase fiber digestibility by at least three different mechanisms: direct stimulation of cellulolytic populations, scavenging of oxygen or stabilization of ruminal pH that favors cellulolytic microorganisms. Chaucheyras et

al. (1995a) demonstrated that SC stimulated germination of *Neocallimastix frontal*is in a vitamin depleted medium, resulting in an increase in cellulose degradation and hydrogen, formate, lactate, and acetate production. Similarly, Dawson et al. (1990) found a stimulatory effect of live yeast on cellulolytic bacteria both *in vivo* and *in vitro*.

In the present experiment, NDF and ADF digestion did not differ ($P > 0.05$) between treatments which is consistent with *in vivo* results reported by Doreau and Jouany (1998) and *in vitro* results from Sullivan and Martin (1999). In ruminants, oxygen enters the rumen via saliva, food, and by diffusion from the blood of the host animal (Czerkawski, 1969). While dissolved oxygen was not determined in the present study, it is likely that most oxygen was displaced by continuous infusion of N_2 which would minimize the potential impact of SC on oxygen scavenging. According to Russell and Wilson (1996), cellulolytic activity is depressed at pH < 6.0, but no differences ($P > 0.05$) in fermentation pH (Table 2.6) were observed in the present study. In addition, Lynch and Martin (2002) reported no change in *in vitro* dry matter degradability (IVDMD) of bermudagrass hay but did find a decrease in IVDMD of alfalfa hay incubated with SC, suggesting that responses may be related to diet.

Effects of SC on VFA concentrations, VFA molar proportions and calculated CH_4 production are depicted in Table 2.4. Addition of yeast resulted in no changes ($P > 0.10$) in total VFA concentrations (m*M*) or in VFA molar proportions (mol/100 mol),

reflecting the lack of differences observed in OM and fiber digestion (Table 2.3). However there was a trend ($P = 0.10$) for YS to decrease A:P ratio because of a numerical decrease in molar proportion of acetate and a numerical increase in propionate. In agreement with the present study, a decrease in A:P ratio following addition of yeast was previously reported from *in vivo* and *in vitro* studies (Williams et al., 1991; Miller-Webster et al., 2002; Guedes et al., 2008; Bagheri et al., 2009). Conversely, Doreau and Jouany (1998) reported no difference in A:P and only transient changes in VFA concentrations in lactating dairy cows. Brossard et al. (2006) found no effect of active dry yeast on rumen VFA from sheep receiving 1x or 10x the recommended commercial dose. In addition, Thrune et al. (2009) reported a trend in total VFA decrease and an increase in molar proportion of butyrate in late lactation dairy cows, probably related to the short adaptation period (6 d) conducted in their experiment. While length of adaptation period was similar in the present experiment (7 d), if the effect of yeast on VFA is transient, these changes may not be detected in continuous culture fermenters when samples are composited daily.

Addition of yeast did not affect ($P < 0.05$) CH_4 production (Table 2.4) which averaged 248.1 and 233.2 ± 10.6 mmol, for NY and YS, respectively. This observation is in agreement with previous *in vivo* (McGinn et al., 2004) and *in vitro* studies (Hristov et al., 2010). Chaucheyras et al. and Mwena et al. (1995b; 2004) reported *in vitro* and *in vivo* decreases in CH_4 production following addition of active yeast. However, in some cases reduction in CH_4 has been associated with a decrease in IVDMD (Lynch and Martin, 2002). While the mechanism involved in CH_4

mitigation by yeast remains obscure, Beauchemin et al. (2009) suggested that an increase in total bacterial numbers and an increase in hydrogen utilization by acetogenic bacteria may be involved. In the present study, no changes in acetate production (Table 2.4) or bacterial N outflow (Table 2.5) were found, supporting the lack of effect of SC on CH_4 production.

Effects of SC on N metabolism are summarized in Table 2.5. Addition of yeast to fermenters reduced ($P < 0.05$) NH_3-N concentration by 37%, with a consequent reduction ($P < 0.05$) in NH_3-N daily outflow. However, daily flows of non NH_3-N, bacterial-N and dietary-N were not altered ($P > 0.05$). Similarly, no effect of SC on CP degradation or efficiency of microbial protein synthesis (EMPS) was observed ($P > 0.05$). The decrease in NH_3-N concentration that was observed in the present study is consistent with previous findings in lactating and non lactating dairy cows (Enjalbert et al., 1999; Moallem et al., 2009), buffalo calves (Kumar et al., 1994), and in gnotobiotically-reared lambs (Chaucheyras-Durand and Fonty, 2001). However, *in vivo* studies from Carro et al. (1992a) showed no differences in rumen NH_3-N and increased duodenal flow of undegraded feed N, probably due to a shorter digesta retention time associated with addition of yeast. Benefits may be obtained if less NH_3-N is produced in the rumen, given the energetic cost of its conversion into urea for further elimination (McBride and Kelly, 1990). In addition, a reduction in NH_3-N may offer environmental benefits because nitrous oxide emitted from urea decomposition has a global warming potential of 296 times that of CO_2 (Steinfeld et al., 2006) and ammonia emissions have been identified as the main cause of

environmental eutrophication (Van Duinkerken et al., 2005). Decreases in NH_3-N may be explained by an inhibitory effect of yeast on the proteolytic population and/or a stimulatory effect on growth of bacterial populations, associated with a higher incorporation of available N into microbial protein, instead of using peptides and amino acids as energy sources (Chaucheyras-Durand et al., 2008). The non-significant, but noticeable reduction in CP degradation obtained in the present study (Table 2.5) supports a reduced proteolytic activity as a more plausible explanation.

Addition of SC to fermenters did not affect ($P > 0.05$) mean and minimum fermentation pH (Table 2.6), averaging 5.74 and 5.68 across treatments, respectively. However, there was a trend ($P = 0.08$) for YS to decrease maximum pH from 5.78 to 5.71. This is probably related to the lower NH_3-N concentration observed for YS compared with NY (Table 2.5). Similar results were reported in batch culture incubations (Lila et al., 2004; Lila et al., 2006), continuous culture incubations (Kung et al., 1997; Miller-Webster et al., 2002) or *in vivo* experiments (Erasmus et al., 2005) using early lactating dairy cows. In contrast, an increase in rumen pH was obtained when active yeast was supplied to late lactation dairy cows under continuous pH monitoring (Bach et al., 2007; Marden et al., 2008; Thrune et al., 2009). Several authors proposed that yeast may increase ruminal pH due to an increase in lactate utilization by *Selenomonas ruminantium* or *Megasphaera elsdenii* (Nisbet and Martin, 1991; Callaway and Martin, 1997), providing a protective benefit against subacute rumen acidosis (SARA). However, SARA can occur because of an increase in rumen VFA concentration rather than lactate

accumulation (Goad et al., 1998). According to Bach et al. (2007), the mechanism by which live yeast ameliorates SARA may be related to meal frequency, but in the present study meal size and frequency were regulated in both treatments. Marden et al. (2008) suggested that yeast can improve conversion of lactate to propionate as a pH stabilization mechanism, different from proton neutralization achieved by conventional buffers like sodium bicarbonate. An additional mechanism has been proposed by Brossard et al. (2006) where *S. cerevisiae* stimulates ciliated protozoa populations that engulf starch, decreasing its availability for lactate-producing amylolytic bacteria. However, this mechanism is difficult to verify in dual flow continuous culture fermenters because they are usually free of protozoa populations (Mansfield et al., 1995; Stern et al., 1997; Ziemer et al., 2000).

The amount of time that fermenter pH was below 5.6, between 5.6 to 5.8 and above 5.8 did not differ between treatments ($P > 0.05$), comprising 1% vs. 0.8%, 78% vs. 89% and 21% vs. 11% of the total time for NY and YS, respectively (Table 2.6). Subacute ruminal acidosis has been characterized as repeated episodes of ruminal pH below 5.6 comprising a total of 3 to 5 h/d (AlZahal et al., 2008). Addition of yeast did not affect ($P > 0.05$) the time spent below the SARA threshold. In the present study, no benefit of yeast supplementation on SARA was obtained. According to Carro et al. (1992b), *in vitro* systems may not be appropriate to study the effect of yeast on pH because of the high buffering capacity provided by artificial saliva.

4. CONCLUSIONS

Addition of active dry yeast to continuous culture fermenters elicited minor changes in fermentation parameters, including a reduction in the maximum pH of fermentations and a trend to reduce A:P ratio.. More importantly, a decrease in NH_3-N concentration and daily outflow suggest that addition of active dry yeast to diets of lactating cows may be beneficial for the host and the environment.

Table 2.1: Ingredient composition of the diet[a] fed to microbes maintained in continuous culture fermenters

Ingredient	% of DM
Corn silage, 40% grain	62.1
Alfalfa hay	13.6
Corn grain, ground	11.3
Soybean meal	6.3
Corn distilled grains	2.9
Molasses	2.8
Blood meal	0.37
Trace minerals[b]	0.15
Limestone	0.14
NaCl	0.14
$CaPO_4$	0.11
MgO	0.09

[a]DM: 56.9%.

[b]Contains (DM basis): Fe, 50 ppm; Zn, 40 ppm; Mn, 40 ppm; Cu, 10 ppm; I, 0.6 ppm; Se, 0.3 ppm; Co, 0.1 ppm.

Table 2.2. Chemical composition of the diet fed to continuous culture fermenters

Item	% of DM
TDN	68.7
NDF	31.6
Roughage NDF	27.1
ADF	20.5
Starch	25.3
CP	17.7
Fat	3.8
NaCl	0.44
P	0.42
Mg	0.33
NEL, Mcal/kg of DM[1]	1.72

[1]Calculated from NRC (2001).

Table 2.3. Effect of active dry yeast (*Saccharomyces cerevisiae*) on organic matter and fiber digestion

	Treatment[1]			
Digestion, %	**NY**	**YS**	**SEM[2]**	***P*-value**
Apparent OM	55.6	56.0	1.6	0.85
True OM[3]	65.6	66.5	0.4	0.24
NDF	51.1	49.4	1.4	0.57
ADF	50.3	48.1	1.4	0.45

[1]NY: no yeast; YS: yeast supplementation.

[2]Standard error of the mean.

[3]Corrected for contribution of microbial OM.

Table 2.4. Effect of active dry yeast (*Saccharomyces cerevisiae*) on VFA concentrations and proportions and methane production in continuous culture fermenters

	Treatment[1]			
Item	**NY**	**YS**	**SEM[2]**	***P*-value**
Total VFA (m*M*)	92.8	93.1	2.18	0.91
Individual VFA (mol/100 mol)				
Acetate	56.0	54.0	0.83	0.13
Propionate	22.7	26.0	1.34	0.13
Butyrate	15.7	14.5	1.03	0.45
Isobutyrate	1.13	1.36	0.35	0.66
Valerate	3.0	2.64	0.18	0.19
Isovalerate + 2-methylbutyrate	0.11	0.10	0.006	0.21
Caproate	1.39	1.49	0.52	0.89
Branched-chain VFA (m*M*)	1.15	1.36	0.64	0.86
A:P Ratio	2.49	2.10	0.14	0.10
CH_4 production (mmol)[3]	248.1	233.2	10.6	0.36

[1]NY: no yeast; YS: yeast supplementation.

[2]Standard error of the mean.

[3]Total mmol produced in 3 days; calculations described by Hegarty and Nolan (2007).

Table 2.5. Effect of active dry yeast (*Saccharomyces cerevisiae*) on nitrogen metabolism

	Treatment[1]			
Item	**NY**	**YS**	**SEM[2]**	***P*-value**
NH_3-N (mg/dL)	6.28	3.85	0.36	0.02
N Flow (g/d)				
NH_3-N	0.16	0.10	0.02	0.04
Non NH_3-N	2.18	2.29	0.06	0.21
Bacterial-N	1.31	1.24	0.07	0.54
Dietary-N	0.88	1.05	0.11	0.30
CP degradation (%)	58.0	50.9	5.05	0.37
EMPS[3]	29.1	25.8	1.61	0.35

[1]NY: no yeast; YS: yeast supplementation.

[2]Standard error of the mean.

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

Table 2.6. Effect of active dry yeast (*Saccharomyces cerevisiae*) on fermentation pH

	Treatment[1]			
Item	**NY**	**YS**	**SEM[2]**	***P*-value**
Mean pH	5.77	5.70	0.03	0.14
Minimum pH[3]	5.70	5.66	0.02	0.18
Maximum pH[3]	5.78	5.71	0.03	0.08
Time below pH 5.6[4]	46.25	33.75	5.8	0.34
Time at 5.6 ≤ pH ≤ 5.8[4]	3368.8	3830.0	202.4	0.31
Time above pH 5.8[4]	905.0	456.3	201.2	0.32

[1]NY: no yeast; YS: yeast supplementation.

[2]Standard error of the mean.

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

[4]Total minutes during 3 consecutive days.

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Chapter 3 : Modification of ruminal and postruminal metabolism by lignosulfonate and polysaccharide protected microminerals measured in vitro

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Abstract. The objective of this experiment was to evaluate the effect of lignosulfonate and polysaccharide protected minerals on *in vitro* rumen fermentation, and ruminal and postruminal partition of Cu, Zn and Mn. Eight dual flow continuous culture fermenters were utilized during two consecutive 10-d periods in a 2 x 2 factorial arrangement of treatments. A synthetic diet consisting of 38% cellulose, 34% starch, 20% powdered whey, 5.3% vegetable oil and 2.5% sugar provided substrate for microbial metabolism. Sulfur was added as $NaSO_4$ or sulfur-bound lignosulfonate to a final concentration of 0.75% of DM. Lignosulfonate was added at 0 (LIG0) or 5% (LIG5) of dry matter. Copper, Zn and Mn were added as $CuSO_4$, $ZnSO_4$ and $MnSO_4$ or as polysaccharide protected Cu, Zn and Mn (SQM- or

SQM+, respectively) to a final concentration of 16, 56 and 71 ppm of DM, respectively. At the end of each period, solid and liquid fractions from fermenters outflows were subjected to pepsin-pancreatin enzymatic digestion. Apparent and true organic matter digestibilities were not affected by treatments. Addition of LIG5 tended to decrease daily flow of non ammonia N, efficiency of microbial protein synthesis, total VFA and molar proportion of acetate, but increased molar proportion of propionate, valerate and caproate whereas SQM+ decreased molar proportion of propionate. Addition of LIG5 increased ruminally soluble Cu and Mn, whereas SQM+ reduced ruminally soluble Cu. Concentration of bacterial Cu and Zn were increased by SQM+ in absence of lignosulfonate. Concentration of Mn was not affected by treatments. Addition of LIG5 resulted in higher enzymatic release of Zn from solids outflow but lower from bacterial pellets. Mean, minimum and maximum fermentation pH were higher with LIG5, and were not affected by mineral source. Addition of lignosulfonate induced major changes in ruminal fermentation. Protected minerals decreased rumen soluble Cu and increased bacterial Cu and Zn without affecting postruminal release of minerals.

Keywords: protected minerals, lignosulfonate, rumen, *in vitro*.

1. INTRODUCTION

Copper, Zn and Mn belong to the group of trace minerals that are essential to ruminants, serving as components of metalloenzymes, enzyme cofactors, and as compounds of hormones. Due to their implication in several physiological processes they play a main role in animal health, growth and productivity (NRC, 2001; Studzinski et al., 2006). While most of the known functions of Cu, Zn and Mn are related to cellular metabolism of the host, some effects on rumen fermentation have been reported (Arelovich et al., 2000; Zhang et al., 2007; Eryavuz and Dehority, 2009). Several interactions in the gastrointestinal tract (i.e., S and Mo with Cu; Ca with Mn; Zn with tannins) have been extensively documented and have usually resulted in decreases in availability of minerals (Sandstrom and Lonnerdal, 1989; Freeland-Graves and Lin, 1991; Underwood and Suttle, 2001; Suttle, 2010).

Lignosulfonate (LIG), a by-product of the paper industry, is a polyphenolic compound derived from lignin. Due to its binding properties LIG is usually used in the manufacturing process of pelleted feeds for animals (Chang et al., 1977). The ability of lignin and lignin by-products to bind minerals such as Cu and Zn has been previously established (Chang et al., 1977; Platt and Clydesdale, 1987). However, the effect of mineral sequestration on rumen metabolism is not fully characterized. In order to minimize mineral binding, protective mechanisms, including chelates, proteinates and polysaccharide complexes are currently available. While the effect of chelates and proteinates has been extensively reviewed (Spears, 1996; 2003; Kinal et

al., 2005), only limited information is available regarding the ruminal effect of polysaccharide complexed minerals (Kennedy et al., 1993; Ruiz-Moreno et al., 2010).

The objective of the present experiment was to evaluate the effect of LIG and a polysaccharide-complex mineral formulation containing Cu, Zn and Mn, on ruminal fermentation and ruminal and postruminal partitioning of Cu, Zn and Mn in outflow fractions using dual flow continuous culture fermenters.

2. MATERIALS AND METHODS

2.1. Experimental diets

Four synthetic diets (Table 3.1) were formulated to meet or exceed the nutritional requirements of a 650 kg Holstein cow producing 32 kg of milk with 3.6% fat and 3.4% protein (NRC, 2001). A basal premix of purified ingredients (cellulose, Solka-floc, International Fiber Corporation, NY; corn starch, Grain Processing Corporation, IO; whey protein isolate, biPro, Davisco Inc., MN) was prepared. Sulfur was added as $NaSO_4$ or sulfur-bound LIG (Ameribond, WI) to a final concentration of 0.75% of DM. Lignosulfonate was added at 0 (LIG0) or 5% (LIG5) of DM. Copper, Zn and Mn were added as $CuSO_4$, $ZnSO_4$ and $MnSO_4$ or as **polysaccharide** protected Cu, Zn and Mn (SQM® protected minerals, QualiTech Inc.; SQM- or SQM+, respectively) to a final concentration of 16, 56 and 71 ppm of DM, respectively. Mixed ingredients were pelleted to a final dimension of 10 x 10 x 5 mm.

2.2. Continuous culture operation

Eight dual flow continuous culture fermenters as described by Hannah et al. (1986) with a modified pH control and measuring system, were randomly assigned to the 4 resulting treatments during 2 consecutive 10-d experimental periods in a 2 x 2 factorial arrangement of treatments. On day 1 of each period, rumen fluid was collected from a cannulated dairy cow fed a high lactation dairy ration (32.7% corn silage; 21.3% lactation protein mix; 19.8% ground corn; 16.1% alfalfa hay; 7.5% fuzzy cottonseed and 2.6% molasses mix) and allocated to each of the eight 1.03 L fermenters. Fermenters were provided with 75 g of DM per day of the diet (Table 3.1), divided in eight equal portions by an automated feeding system. Each portion was provided during a 90-minute period beginning at 0300, 0600, 0900, 1200, 1800, 2100 and 2400 h daily.

Liquid flow rate of fermenters was set at 10%/h by regulating artificial saliva (pH = 8.25) input whereas solids dilution rate was set at 5.5%/h by regulating liquid output through filters. Individual pH was recorded every 5 min by an electronic data acquisition system (Daisy Lab®) and was maintained between 5.5 and 6.5 by automated addition of either 5N NaOH or 3N HCl. Anaerobic conditions were maintained by continuous infusion of N_2 at a rate of 40 mL/min throughout the experiment. Fermenter temperature was maintained at 39.1 ± 0.1°C by an electrical heater.

2.3. Sample collection and analytical procedures

After a 7-d adaptation period, fermenter effluents were maintained at 1 °C in a water bath to retard microbial and enzymatic activities. During three consecutive days, fermenter effluents were homogenized using a PT10/3S homogenizer (Kinematica GmbH, Bohemia, NY), and 500 mL aliquots were removed daily. Separate composites of effluents and fermentor contents were composited by fermenter. Subsamples from liquid effluent were centrifuged at 30,000 x *g* for 30 min and stored at -20 °C for further mineral analysis. Subsamples from solid fermenter effluent and the microbial pellet were recovered for post-ruminal digestion and for mineral analysis as depicted in Figure 3.1. At the end of each experimental period, contents from each fermenter were strained through two layers of cheesecloth, centrifuged at 1,000 x *g* for 10 min to remove feed particles and the supernatant was centrifuged at 20,000 x *g* for 20 min to isolate bacteria. Bacterial pellets were resuspended in distilled water, frozen and lyophilized. Freeze-dried composite samples were analyzed for DM, OM, NDF, AFD, ash, and purines. Composite effluent samples were analyzed for total-N, ammonia-N (NH_3-N) and VFA.

2.4. Postruminal simulation

The two solids fractions recovered from the fermenters (solid outflow, and bacterial pellet) were subjected to an enzymatic digestion procedure similar to that of Calsamiglia and Stern (1995). The aim of this procedure was to simulate abomasal and small intestinal conditions. In brief, samples (containing 15 mg N)

were incubated in a solution containing 1 g/L pepsin (pH = 1.9) during 1 h at 38.6°C. Afterwards, a 24 h incubation in a solution containing buffered pancreatin (3 g/L; pH = 7.8; 38.6°C.) was conducted. Following incubations, supernatant was recovered, centrifuged at 30,000 x *g* for 30 min and stored at -20 °C for mineral analysis.

2.5. Chemical analyses

Dry matter content of diets, outflows and microbial pellets was determined by drying in an oven at 105°C for 24 h. Ash was determined by weight difference after 24 h combustion at 550 °C (AOAC, 1984). Purine concentrations were determined by the method of Zinn and Owens (1986). Purine content of effluent and bacteria was used to partition flow of effluent N into microbial and dietary N. Total N in the effluent, bacteria, and diet was determined (AOAC, 1984). Ammonia-N was determined by steam distillation (Bremner and Keeney, 1965) using a Kjeltech 2300 Analyzer Unit (Tecator, Herdon, VA). Sequential detergent fiber analyses (Van Soest et al., 1991) were used to determine NDF and ADF concentrations of diet and effluents. Effluent VFA concentrations were determined by capillary GC analysis. Incubation fluid was solvent-extracted using ethyl acetate (3:7 ratio) during 10 minutes under continuous vortex. Samples were centrifuged at 5,000 x *g* for 5 min and supernatant was stored at -20 °C until analyzed. Analysis was performed using a Hewlett-Packard 5890 GC equipped with a Stabilwax-DA capillary column (30 m x 0.25 mm i.d., 0.25 μm film thickness, Restek, CA). Chromatographic conditions were: helium (1.7 mL/min), initial oven temperature, 110°C, held for 2.1 min; ramped at

25°C/min to 200°C, held for 1.5 min; injector temperature: 200°C, flame ionization detector temperature: 220°C; split injection (split ratio: 1/10); injection volume: 1 μL. Ethyl butyrate was included as an internal standard.

Mineral analysis (Cu, Zn, Mn) was conducted by ICP-AES on a Perkin Elmer Optima 3000 ICP. Fermenter outflow and microbial pellet samples were ashed at 485°C for 10 h and dissolved in 10% HCl. Liquid samples (centrifuged fraction) were diluted in nanopure water to fit in the instrument calibration range.

2.6. Statistical analyses

Data were analyzed as a 2 x 2 factorial arrangement of treatments in a randomized complete block design. The model for each dependent variable was:

$$Y_{ij} = \mu + B_i + P_j + M_k + P_j \times M_k + \varepsilon_{ijk}$$

where μ is the grand mean, B_i is the period (block), P_j is the LIG level, M_k is the mineral source, P_j x M_k is the interaction term and ε_{ijk} is the random error. Differences were considered significant at $P \leq 0.05$ and tendencies discussed at $P \leq 0.1$. Data for fermentation pH that was obtained on 3 consecutive days were analyzed as a repeated measures analysis of variance with an autoregressive order 1 structure of covariance, based on minimum values of AIC (Akaike's Information Criterion). Lowest and highest hourly recorded pH were used for minimum and maximum pH comparison between treatments. Time (min) spent below pH 5.6, between pH 5.6 to 5.8 and above pH 5.8 was calculated using the trapezoidal integration method. Data processing was conducted using the statistical software R

(R Development Core Team, 2005). Unless noted otherwise, results are reported as least squares means from 4 observations/treatment.

3. RESULTS AND DISCUSSION

Apparent and true organic matter digestion was not affected ($P < 0.05$) by addition of LIG or mineral source, averaging 41.1 and 41.2%, respectively (Table 3.2). However, LIG increased ($P < 0.05$) ADF digestion by 14%, whereas SQM+ minerals decreased ($P < 0.05$) NDF and ADF digestion by 13.6 and 11%, respectively. These results partially agree with *in vitro* observations from Chang et al. (1977) who reported a decrease in dry matter digestion of soybean meal and alfalfa following the addition of LIG (4.56% DM basis), but not when bleached paper was used as the incubation substrate and 6.84% LIG (DM basis) was added, indicating a role of dietary composition on LIG effect. Stern (1984) reported no changes in true OMD and cellulose digestion in continuous culture fermenters when Ca-lignosulfonate was added at 4% of DM, but digestion of true OM and cellulose was depressed the addition of Ca-lignosulfonate at 8% of DM. The increase in ADF digestion in the present experiment is difficult to explain, however factors other than lignosulfonic acid present in lignosulfonate may be related to the observed results (Windschitl and Stern, 1988). Russell and Dombrowski (1980) established that rumen cellulolytic populations are sensitive to low pH, with minimum pH for growth being about 5.9. In the present study, addition of LIG5 resulted in higher pH

than LIG0 (Table 3.8) and the minimum pH matched the critical value for cellulolytic populations established by Russell and Dombrowski (1980). These results indicate addition of LIG provided a more friendly pH environment for cellulolytic bacteria which supported ADF and NDF digestion.

The role of trace minerals on rumen fiber digestion is not clearly established. Early studies of Martinez and Church (1970) examined different concentrations of trace minerals on purified cellulose and showed that 0.01, 5 and 15 ppm of Cu, Zn and Mn respectively, resulted in the greatest cellulose digestion. More recently, Eryavuz and Dehority (2009) showed that 50 to 150 ppm Zn caused a decrease in *in vitro* cellulose digestion, but no differences were found at lower Zn concentrations. Inconsistent results have been reported on the role of Cu on fiber digestion. Zhang et al. (2009) found an *in vivo* decrease in NDF digestion when Cu was added to the diet of cashmere goats at more than 35.6 ppm but not with lower concentrations. In the same study, ADF digestion was not affected by Cu levels. However in a previous study (Zhang et al., 2007), the addition of 17 or 27 ppm Cu increased *in vivo* NDF digestion. Arelovich et al. (2000) reported an increase in IVDMD of prairie hay following the addition of 100 ppm of Mn, compared with 0 ppm, but no other reports on the role of Mn on fiber digestion are available. In the present study, SQM+ decreased ($P < 0.05$) the concentration of Cu in the ultracentrifugate from vessels (Table 3.5) and simultaneously decreased NDF and ADF digestion. These results are in contrast to the relationship between Cu and cellulolytic activity reported by Martinez and Church (1970). However, the soluble Cu concentrations obtained in

the current study are much lower than those reported by Martinez and Church (1970) and thus should not be inhibitory for cellulolytic activity.

Effects of treatments on VFA metabolism are shown in Table 3.3. Addition of LIG resulted in a 12% decrease ($P < 0.05$) of total VFA concentration, along with a decrease ($P < 0.05$) in acetate molar proportion. Conversely, an increase ($P < 0.05$) in molar proportion of propionate, valerate and caproate was observed. However, these differences did not affect ($P = 0.18$) the A:P ratio. No differences ($P > 0.05$) were obtained for molar proportions of isobutyrate or isovalerate + 2-methylbutyrate as well as for total branched-chain VFA concentration. Similar reduction in total VFA was found by Windschitl and Stern (1988) in Holstein cows, when comparing the use of unprotected soybean meal with lignosulfonate-treated soybean meal. This reduction can be explained by a decrease in rumen CP digestion found in their study, but no differences in CP digestion were obtained in our study (Table 3.4). In agreement with the present results, a decrease in total VFA and acetate concentration was found by Chang et al. (1977) following the addition of lignosulfonate at 8% of DM to the diet of feedlot steers. However, total VFA concentration was not affected by lignosulfonate added at 4% of DM, and was maximized when added at 12% of DM. This effect is not unique to ruminants because Ricke et al. (1982) found lower concentrations of cecal and large intestinal VFA in chicks fed lignin than in those fed a control diet, and is probably related to the antimicrobial properties of several lignin fragments as previously demonstrated by Zemek et al. (1979).

In contrast to LIG, protected minerals increased ($P < 0.05$) the molar proportion of acetate and decreased ($P < 0.05$) propionate, without affecting ($P > 0.05$) the molar proportion of other VFA. Concentration of branched chain VFA and A:P ratio did not differ ($P < 0.05$) between the mineral sources. Information regarding the effect of organic minerals on VFA production is limited. Spears et al. (2004) found a reduction in total VFA when Zn was provided to steers as Zn-methionine or Zn-glycine, compared with $ZnSO_4$. In addition the authors found an increase in molar proportion of propionate with the consequent decrease in the A:P ratio, but molar proportions of butyrate and valerate remained unchanged, in partial agreement with the present study. However, no direct comparison between studies is possible because VFA concentration was measured at 2 h post feeding, as opposed to the continuous collection conducted in the current study. In another study, Mandal et al. (2007) found that addition of Zn-propionate did not affect rumen VFA, compared with $ZnSO_4$. Similarly, no effect of Zn-methionine or Zn-lysine on rumen VFA was noted by Chen et al. (2001) in Holstein–Friesian cattle.

Table 3.4 shows the effect of treatment on nitrogen metabolism. Ammonia N concentration was extremely high for all treatments and in excess of the minimum proposed by Satter and Slyter (1974) to ensure microbial growth. However, no effect ($P > 0.05$) of LIG or mineral source was found, averaging 48.4 mg/dL across treatments. Consistent with this observation, no differences ($P > 0.05$) were obtained in daily NH_3-N flow, but addition of LIG resulted in a trend ($P < 0.10$) for a lower non NH_3-N flow. This trend can be related to a numerical decrease ($P > 0.05$)

obtained for bacterial-N and dietary-N daily flows obtained following the addition of LIG, because no differences ($P > 0.05$) between treatments were noticed for CP degradation. Traditionally, addition of lignosulfonate results in a decrease in rumen CP degradation (Calsamiglia et al., 1995; Von Keyserlingk et al., 2000; Borucki Castro et al., 2007), however for the protective effect to occur, temperature must be applied during the processing of the dietary protein source (Wright et al., 2005). The lack of protection in the present experiment is not surprising because temperature was not applied during manufacturing of the pelleted diets, in order to isolate the effect of lignosulfonate on rumen fermentation rather than effects resulting from lignosulfonate and protein interactions. Addition of LIG resulted in a marked reduction ($P < 0.05$) in efficiency of microbial protein synthesis (Table 3.4) probably related to role of phenolic monomers in altering microbial cell metabolism as previously established by Borneman et al. (1986). Conversely, no effects ($P < 0.05$) of mineral source were obtained on N metabolism. Similarly, Mandal et al. (2007) found no differences in nonprotein N and NH_3-N rumen concentration of cows receiving organic or inorganic sources of Zn. In support of this observation, a previous study by Kincaid et al. (1997) found no differences in rumen N metabolism when comparing Zn supplementation as Zn methionine, Zn lysine, ZnO or $ZnSO_4$ to Holstein calves.

Table 3.5 shows the effect of LIG and mineral source on soluble Cu, Zn and Mn in fermentation vessels. Addition of 5% LIG resulted in 39 and 15% greater ($P < 0.05$) solubilization of Cu and Mn, respectively, without affecting ($P > 0.05$)

concentration of soluble Zn. While these results were unexpected, they can be partially related to differences in ADF digestion observed following addition of LIG (Table 3.2). According to Idouraine et al. (1995), ADF has the ability to bind Cu and Zn, with binding capacity highly variable, depending on chemical composition and structure of fiber. In addition, Thompson and Weber (1981) found that cellulose can bind Cu and Zn, having more affinity for Cu. In the present experiment, purified cellulose was the only source of dietary fiber. It is possible that an increase in cellulose digestion decreased mineral binding capacity for Cu and Zn, resulting in higher concentrations of Cu and Zn in the soluble fraction of the vessels.

No effect ($P > 0.05$) of mineral source on soluble concentrations of Zn and Mn were noted (Table 3.5). In a study comparing polysaccharide protected Zn with ZnO, Kennedy et al. (1993) found higher Zn solubilization with ZnO at 2 and 4 h post administration, but not at 6 or 8 h. Spears et al. (2004) reported higher rumen soluble Zn following administration of Zn-methionine, but not that of Zn-glycine compared with $ZnSO_4$. These results indicate that protective mechanism and sampling time may be relevant for rumen Zn solubilization. In contrast, addition of polysaccharide protected minerals resulted in 17% ($P < 0.05$) reduction in soluble Cu (Table 3.5). A lower solubilization can be interpreted as a binding of Cu with dietary compounds (fiber, protein), however another explanation implies that, in contrast to $CuSO_4$, a higher proportion of Cu remained bound to the polysaccharide, being protected from potential rumen interferences. Underwood and Suttle (2001) recommended that organic mineral formulations should be compared with inorganic

sulfates of the same mineral, given their high availabilities in the rumen. Current results suggest that polysaccharide protected Cu provides a protective mechanism for Cu compared with $CuSO_4$.

Table 3.6 shows the effect of treatments on Cu, Zn and Mn in bacterial pellets. An interaction between treatments was found for bacterial Cu and Zn content. In the absence of LIG, protected minerals increased ($P < 0.05$) bacterial Cu and Zn concentration by 56 and 45%, respectively, but no differences ($P > 0.05$) were obtained following addition of LIG, averaging 33.2 and 106.5 ppm for Cu and Zn, respectively. Manganese concentration in bacterial pellets was not affected ($P > 0.05$) by LIG or mineral source, averaging 93.8 ppm across treatments. Copper concentration found in the present study is in agreement with a previous study from Van Ryssen et al. (1998) who found values ranging from 28.7 to 38.5 ppm of Cu in rumen bacteria of ewes receiving different levels of dietary Se. Hartmann and van Ryssen (1997) reported similar values ranging from 14 to 32 ppm of Cu in bacterial DM, depending on the dietary Cu concentration. In the present study, concentration of Cu in rumen bacteria was 2.1 to 4.8 higher than dietary Cu. Zinc concentration in bacteria ranged from 1.7 to 3.2-fold higher than dietary Zn, indicating that the dietary minerals had been incorporated or associated with microbial cells. However, bacterial Mn concentration resulted only in 1.1 to 1.5 times that of dietary Mn. Similar to the present study, Kennedy et al. (1993) found higher concentration of Zn in rumen bacterial cells than in the diet, and no differences were found when Zn was provided as Zn-oxide or as a Zn-polysaccharide. According to Durand and

Kawashima (1980), microbial Zn can be 10 to 500% higher than dietary Zn. The same authors established that Mn concentration in rumen bacteria was approximately 34 to 38% higher than in the diets, which agrees with the 32% found in the present study.

The proportion of Cu, Zn and Mn released from solids outflows and bacterial pellets following pepsin-pancreatic digestion is shown in Table 3.7. No differences ($P > 0.05$) between treatments were obtained in the release of Cu from solid outflows, averaging 53.4% of the Cu exposed to digestion. However, addition of LIG increased ($P < 0.05$) the release of Zn without affecting ($P > 0.05$) that of Mn. Conversely, the enzymatic release of Zn was not affected ($P > 0.05$) by the mineral source, but a trend ($P < 0.1$) for a higher release of Mn was obtained following the addition of polysaccharide protected minerals to the diet. No detectable concentrations of Cu were found after the enzymatic digestion of bacterial cells. However, released Zn was 34% lower ($P < 0.05$) in the presence of LIG, without being affected ($P > 0.05$) by mineral source. In addition, the proportion of Mn released from digested bacterial cells was not affected by treatments, averaging 48% of the total Mn (Table 3.7). To the best of our knowledge the current study is the first one to evaluate the effect of protected minerals on mineral composition of rumen bacteria.

Addition of LIG at 5% DM resulted in a greater ($P < 0.05$) mean, minimum and maximum pH of fermentations, compared with 0% LIG (Table 3.8). This was partially achieved by a shift in the amount of time spent at the different pH

intervals. While the addition of 5% LIG resulted in a 48% reduction of time spent between pH 5.6 to 5.8, time spent above 5.8 increased by 54% and no differences ($P > 0.05$) were obtained for time spent under pH 5.6, representing 5.8 and 8.6% of total incubation time for 0 and 5% LIGN, respectively. The lower pH obtained without LIG can be explained by the higher ($P < 0.05$) total VFA concentration found with LIGN at 0% compared with LIGN at 5% (Table 3.3). These results are consistent with those from Castillejos et al. (2006) who showed a decrease in *in vitro* VFA concentrations associated with an increase in rumen pH. A similar relationship was found *in vivo* by Windschitl and Stern (1988) after replacing soybean meal with lignosulfonate-treated soybean meal in the diet of lactating cows. Conversely to LIG, mineral source did not affect ($P < 0.05$) fermentation pH, averaging 5.9, 5.8 and 6.0 for mean, minimum and maximum pH, respectively. Consistent with these observations, no differences ($P > 0.05$) were obtained in the amount of time spent at different pH intervals, representing 7.2, 40.1 and 52.7% of the incubation for time spent below 5.6, between 5.6 to 5.8 and above 5.8, respectively (Table 3.8). The lack of differences in pH can be explained by lack of effect of mineral source on VFA and NH_3-N (Table 3.3 and Table 3.4, respectively). Similarly, no differences in rumen pH were found *in vivo* when increasing concentrations of Zn were added to the diet of steers (Arelovich et al., 2008), or when organic and inorganic sources of Zn were studied (Mandal et al., 2007). In addition, Engle and Spears (2000) found no effect of increasing amounts of dietary Cu on rumen pH.

4. CONCLUSIONS

Major changes in rumen metabolism were induced by addition of lignosulfonate, including an increase in fiber digestion, reductions in VFA production and EMPS, and an increase in fermentation pH. According to these findings, lignosulfonate has the ability to modify rumen fermentation beyond its conventional use as a protein protecting agent. Polysaccharide protected minerals did not have a substantial effect on rumen fermentation. Copper solubilization was lower with polysaccharide protected Cu, suggesting a rumen protective mechanism. In absence of lignosulfonate, polysaccharide protected minerals resulted in higher Cu and Zn bacteria uptake. After enzymatic digestion of solid outflows and bacterial pellets, mineral release was not different between mineral sources. In the current experiment, the ability of polysaccharide complexes to protect microminerals appears to be effective in absence of lignosulfonate only. Further research is necessary to develop mineral protecting technologies able to overcome dietary antagonisms in ruminants.

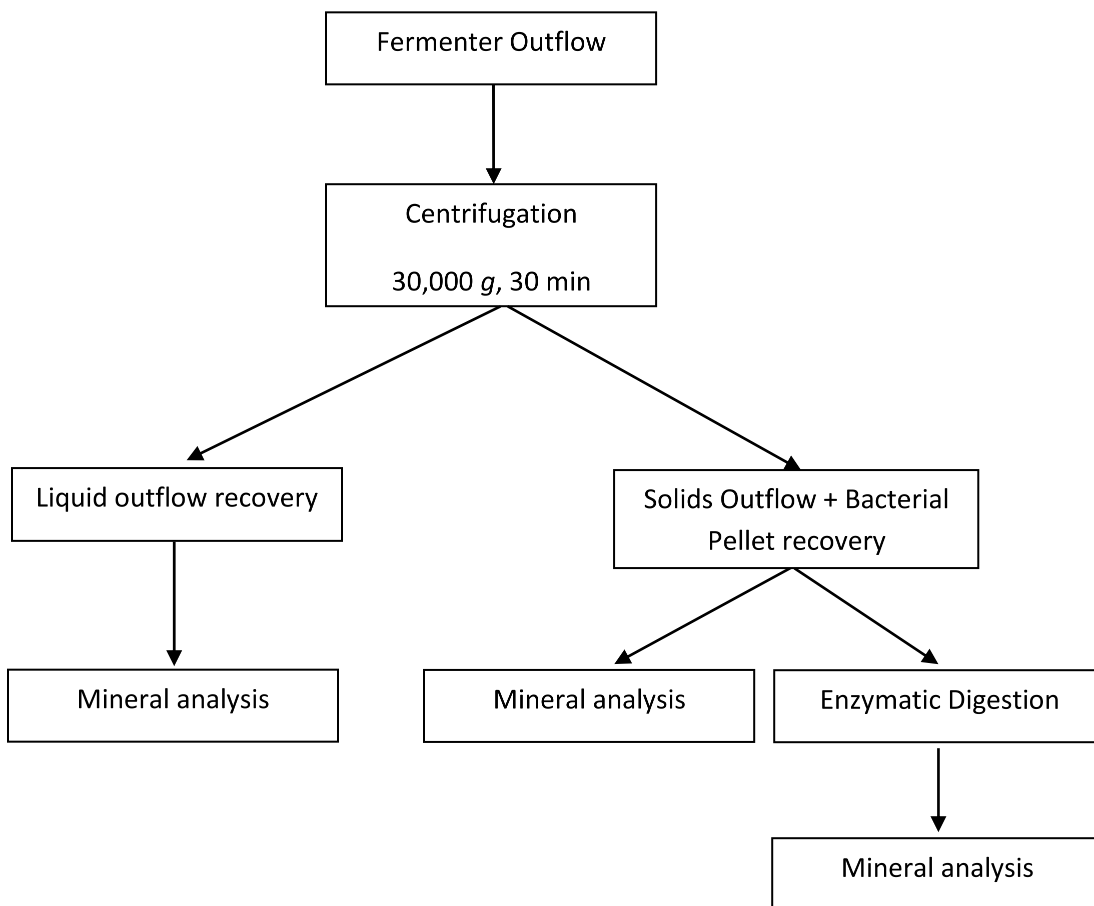

Figure 3.1: Scheme of sample processing for mineral analysis

Table 3.1: Ingredient and chemical composition of experimental diets

	Diet[1]			
Ingredient, % of DM	**L+SQM-**	**L-SQM-**	**L+SQM+**	**L-SQM+**
Cellulose[2]	36	36	36	36
Starch[3]	32	32	32	32
Protein[4]	19	19	19	19
Molasses	4	4	4	4
Fat	5	5	5	5
Lignosulfonate[5]	5.95	-	5.95	-
Micromineral mix	0.03[6]	0.03[6]	0.03[7]	0.03[7]
Chemical composition	------------------------- of DM --------------------------			
CP	17.0	15.9	17.2	17.3
ADF	25.7	28.6	31.0	29.5
NDF	32.5	35.1	36.6	33.4
TDN	76.0	75.5	75.0	76.0
NEL, Mcal/Lb	0.82	0.81	0.80	0.82
NEM, Mcal/Lb	0.84	0.83	0.82	0.84
NEG, Mcal/Lb	0.56	0.54	0.54	0.55
Ca	1.18	1.36	0.93	0.95
P	0.46	0.50	0.46	0.48
Mg	0.32	0.36	0.35	0.35
K	1.22	1.30	1.21	1.26
Na	0.71	0.77	0.73	0.74
S	0.90	0.94	0.60	0.61
	-------------------------- ppm --------------------------			
Fe	83.0	70.0	70.5	69.5

Zn	51.5	58.5	55.0	57.5
Cu	17.5	19.0	13.5	14.5
Mn	71.0	80.0	67.0	67.0
Mo	0.90	0.70	0.95	0.95

[1]L+SQM-: 5% lignosulfonate, non-protected minerals; L-SQM-: 0% lignosulfonate, non-protected minerals; L+SQM+: 5% lignosulfonate, rumen-protected minerals; L-SQM+: 0% lignosulfonate, rumen-protected minerals.

[2]Solka-floc (International Fiber Corporation, NY).

[3]Corn starch (Grain Processing Corporation, IO).

[4]Whey protein isolate (biPro, Davisco Inc., MN).

[5]Lignosulfonate (Ameribond, WI., containing: Ca, 7.51%; P, .05%; S, 6.96%; Mg, .08%, K, .15%; Na, .29%, Fe, 78 ppm; Zn, 46 ppm; Cu, 106 ppm; Mn, 316 ppm; Mo, .2 ppm; Co, 3.95 ppm.).

[6]Formulated with inorganic Cu, Zn and Mn.

[7]Formulated with SQM® minerals (QualiTech Inc).

Table 3.2: Effect of lignosulfonate and protected minerals on organic matter and fiber digestion

	LIG		Minerals			*P*-value		
Digestion	0%	5%	SQM-	SQM+	SEM[1]	LIG	SQM	L*SQM
OMD, apparent	40.1	42.1	41.2	41.0	0.9	0.13	0.88	0.65
OMD, true[2]	46.9	47.3	47.1	47.2	0.7	0.12	0.94	0.73
NDF	30.8	33.4	34.4	29.7	1.3	0.18	0.02	0.92
ADF	31.4	35.8	35.7	31.6	1.4	0.04	0.05	0.45

[1]Standard error of the mean.

[2]Corrected for contribution of bacterial OM.

Table 3.3: Effect of lignosulfonate and protected minerals on volatile fatty acid metabolism

	LIG		Minerals			*P*-value		
Item	0%	5%	SQM-	SQM+	SEM[1]	LIG	SQM	L*SQM
Total VFA (m*M*)	66.4	58.3	63.0	61.8	2.4	0.04	0.72	0.19
Individual VFA (mol/100 mol)								
Acetate	68.2	56.8	58.7	66.3	2.5	0.009	0.06	0.94
Propionate	27.7	36.8	35.5	28.4	2.3	0.03	0.05	0.84
Butyrate	2.47	3.82	3.48	2.81	0.57	0.13	0.43	0.93
Isobutyrate	0.20	0.35	0.32	0.24	0.07	0.17	0.44	0.52
Valerate	0.90	1.81	1.21	1.50	0.25	0.03	0.43	0.42
Isovalerate + 2-methylbutyrate	0.42	0.71	0.59	0.53	0.12	0.12	0.72	0.39
Caproate	0.10	0.24	0.14	0.20	0.03	0.02	0.24	0.15
Branched-chain VFA (m*M*)	0.42	0.63	0.58	0.50	0.12	0.22	0.64	0.80
A:P Ratio	2.82	1.82	1.91	2.74	0.41	0.18	0.25	0.40

[1]Standard error of the mean.

Table 3.4: Effect of lignosulfonate and protected minerals on nitrogen metabolism

	LIG		Minerals			*P*-value		
Item	0%	5%	SQM-	SQM+	SEM[1]	LIG	SQM	L*SQM
NH_3-N (mg/dL)	38.5	58.3	45.1	51.7	12.06	0.28	0.71	0.66
N flow (g/d)								
Ammonia-N	0.92	1.36	1.08	1.21	0.42	0.32	0.76	0.68
Non ammonia-N	2.35	1.98	2.17	2.15	0.13	0.07	0.94	0.81
Bacterial-N	1.21	1.13	1.15	1.18	0.04	0.17	0.61	0.93
Dietary-N	1.13	0.85	1.02	0.97	0.16	0.22	0.84	0.85
CP degradation (%)	44.6	43.5	37.8	50.2	7.97	0.93	0.30	0.25
EMPS[2]	21.2	15.2	17.4	19.0	1.30	0.006	0.40	0.57

[1]Standard error of the mean.

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

Table 3.5: Effect of lignosulfonate and protected minerals on Cu, Zn and Mn solubilized in vessels and daily flow

	LIG		Minerals			*P*-value		
Mineral	0%	5%	SQM-	SQM+	SEM[1]	LIG	SQM	L*SQM
	----------------ppm[2]----------------							
Cu	0.23	0.32	0.30	0. 25	0.02	0.008	0.04	0.33
Zn	0.67	0.67	0.69	0.65	0.05	0.91	0.49	0.50
Mn	1.80	2.07	1.99	1.88	0.05	0.003	0.13	0.11

[1]Standard error of the mean.

[2]Obtained at 30,000 *g* for 30 minutes.

Table 3.6: Effect of lignosulfonate and protected minerals on Cu, Zn and Mn concentration in bacterial pellets[1]

Mineral	Treatment[2]				SEM[3]	*P*-value		
	L-SQM-	L-SQM+	L+SQM-	L+SQM+		LIGN	SQM	L*SQM
Cu	44.9[b]	70.2[a]	36.4[b]	29.9[b]	6.9	0.004	0.20	0.04
Zn	126.2[b]	182.8[a]	116.0[bc]	97.0[c]	9.4	0.0003	0.07	0.0017
Mn	88.1[a]	103.8[a]	88.3[a]	94.1[a]	9.9	0.64	0.30	0.63

[1]ppm, DM basis.

[2]L-SQM-: 0% lignosulfonate, non-protected minerals; L-SQM+: 0% lignosulfonate, rumen-protected minerals; L+SQM-: 5% lignosulfonate, non-protected minerals; L+SQM+: 5% lignosulfonate, rumen-protected minerals.

[3]Standard error of the mean.

[a, b]Means in the same row with unlike superscripts differ ($P < 0.05$).

Table 3.7: Copper, zinc and manganese released from solid outflows and bacterial cells after pepsin-pancreatin digestion[1]

Mineral	LIG		Minerals		SEM[2]	*P*-value		
	0%	5%	SQM-	SQM+		LIG	SQM	L*SQM
	From solid outflows							
Cu	57.9	49.0	51.9	54.9	5.1	0.25	0.68	0.86
Zn	19.2	23.5	20.4	22.3	1.4	0.05	0.35	0.06
Mn	13.0	11.3	11.1	13.1	0.8	0.14	0.09	0.51
	From bacterial cells							
Cu	ND[3]	ND[3]	ND[3]	ND[3]	-	-	-	-
Zn	53.1	33.6	37.9	48.8	9.4	0.05	0.27	0.40
Mn	52.7	43.2	42.4	53.7	11.2	0.56	0.50	0.90

[1]Percentage of Cu, Zn, Mn exposed to pepsin-pancreatin.

[2]Standard error of the mean.

[3]ND: non-detectable levels.

Table 3.8: Effect of lignosulfonate and protected minerals on fermentation pH

	LIG		Minerals			*P*-value		
pH	**0%**	**5%**	**SQM-**	**SQM+**	**SEM[1]**	**LIG**	**SQM**	**L*SQM**
Mean	5.82	6.00	5.91	5.91	0.02	0.003	0.83	0.79
Minimum	5.68	5.92	5.83	5.77	0.06	0.04	0.48	0.50
Maximum	5.93	6.09	6.01	6.01	0.02	0.004	0.87	0.49
Time below 5.6[2]	248.5	370.4	389.1	229.8	217.9	0.70	0.62	0.11
Time at 5.6 to 5.8[2]	2258	1178	1687	1748	155.1	0.001	0.75	0.52
Time above 5.8[2]	1779	2737	2209	2307	202.1	0.01	0.74	0.08

[1]Standard error of the mean.

[2]Minutes over 3 days.

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Chapter 4：In vitro mitigation of rumen hydrogen sulfide

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Abstract. Two *in vitro* experiments were conducted to assess the effects of bismuth subsalicylate (BSS) and monensin (MON) on rumen metabolism and H_2S release by rumen microbes. In experiment 1, two consecutive 24-h batch culture incubations were conducted using a diet consisting of 50% ground corn, 40% distiller grains, 9.75% hay and 0.25% mineral premix, on a DM basis. Rumen fluid was obtained from a lactating cannulated dairy cow and mixed with McDougall's saliva to a 1:2 ratio. Treatments were designed so that BSS was added to final concentrations of 0 (control), 0.5, 1, 2 and 4% of DM, and were assigned in 5 replicates to 120-mL serum bottles containing 50 mL of the inoculum mix and 0.5 g of diet. Incubations were conducted at 39.1°C. Final pH increased ($P < 0.05$) with 2 and 4% BSS treatments. There was no effect ($P > 0.05$) of BSS on NH_3-N concentration. At 4% of DM, BSS decreased ($P < 0.05$) total VFA concentration (m*M*) and molar proportion (mol/100 mol) of propionate while increasing acetate proportion and A:P ratio. Molar

proportion of butyrate was lower with 2% BSS than the control ($P < 0.05$). Concentration of branched-chain VFA increased ($P < 0.05$) with the addition of 0.5% BSS, compared with the control. Compared with the control, gas production decreased ($P < 0.05$) with the 2 and 4% BSS treatments. All concentrations of BSS reduced ($P < 0.05$) H_2S production by 18, 24, 82 and 99% for 0.5, 1, 2 and 4% BSS, respectively. Based on these results, addition of BSS at 1% of DM and MON (5 ppm) were used to assess their effect on metabolism and H_2S release by rumen microbes maintained in long term continuous incubations (Experiment 2). Eight dual flow continuous culture fermenters were used during 2 consecutive 10-d periods, in a 2 x 2 factorial arrangement of treatments. Incubation substrate consisted of 46% distillers grains, 41% ground corn, 8% hay, 2.5% $CaCO_3$, 1.5% molasses and 1% mineral premix and was provided at 75 g/ferm/d. Liquid and solids outflow rates were set at 8.2 and 4.1%/h, respectively. Incubations were conducted at 39.1°C. Addition of BSS to the diet increased ($P < 0.05$) digestion of OM, NDF and ADF but decreased ($P < 0.05$) that of NFC and total VFA concentrations. Molar proportions of acetate and propionate increased ($P < 0.05$) with BSS in the diet, while that of butyrate was decreased ($P < 0.05$). Monensin decreased ($P < 0.05$) ADF digestion and A:P ratio, without affecting molar proportions of major VFA. Regarding nitrogen metabolism, MON increased ($P < 0.05$) non NH_3-N outflow without affecting ($P > 0.05$) other measurements. Addition of BSS to the diet increased ($P < 0.05$) NH_3-N concentration, NH_3-N flow and dietary-N flow, while decreasing ($P < 0.05$) microbial-N outflow, CP digestion, and efficiency of microbial protein synthesis. Headspace H_2S was reduced ($P < 0.05$) by 99% with BSS treatment but was not affected ($P >$

0.05) by MON. Only minor changes in fermentation pH were found with MON, but an increase ($P < 0.05$) in mean, minimum and maximum fermentation pH were found following addition of BSS. Results indicate that BSS can markedly reduce H_2S production in short term and long term *in vitro* rumen incubations.

Keywords: Hydrogen sulfide, rumen, bismuth subsalicylate

1. INTRODUCTION

In ruminants, an excess of dietary S is associated with several conditions including reduced dry matter intake (Bouchard and Conrad, 1974; Drouillard et al., 2009), negative effects on feedlot performance and carcass characteristics (Loneragan et al., 2001), and sulfur-associated polioencephalomalacia (PEM) (Gould, 1998). Polioencephalomalacia has been related to high S levels in water (Haydock, 2003), and feed sources (Niles et al., 2000). While PEM has been extensively documented in feedlot animals (Gould et al., 1997; Vasconcelos and Galyean, 2008), the disease has also been observed in lactating dairy cows (Kul et al., 2006) and sheep (Rousseaux et al., 1991). Previous studies conducted by Gould et al. (1997; 1991) demonstrated a positive association between elevated hydrogen sulfide (H_2S) concentrations in rumen fluid and rumen gas cap, and onset signs of PEM in steers. Through adaptation, rumen microbial populations appear to have a role in conversion of dietary sulfur into sulfide (Cummings et al., 1995). The use of antibiotics to modulate H_2S production has been tested with controversial results in short term rumen fluid incubations. Kung et al. (2000) found an increase in H_2S concentration following addition of monensin, but no effect of monensin and other ionophores on H_2S was obtained by Quinn et al. (2009) in batch culture incubations.

During the last decade, the use of corn distillers dry grains as an ingredient in ruminant diets has increased and is currently included in rations as a primary rather than a secondary energy source (Depenbusch et al., 2009). Distiller dry grains

can contain significant amounts of S (Crawford, 2007; Schingoethe et al., 2009) and have been associated with PEM (NRC, 2001).

In humans, H_2S has been associated with pathogenesis of ulcerative colitis (Rowan et al., 2009) and many cationic compounds have shown the ability to reduce *in vitro* H_2S concentrations (Mitsui et al., 2003). Among them, bismuth subsalicylate (BSS) seems promising because it can markedly reduce H_2S concentrations without affecting the normal flora of the intestinal tract (Suarez et al., 1998a). However, no studies exploring the effect of BSS on rumen fermentation have been conducted.

The objective of the present study was to establish the effect of different doses of BSS on H_2S release and rumen fermentation in batch cultures, and to evaluate its effects in association with monensin on H_2S release and rumen fermentation parameters in long term continuous culture incubations.

2. MATERIAL AND METHODS

The present study was divided into two experiments. The first experiment consisted of a short term batch culture incubation where the effect of different doses of BSS (ranging from 0 to 4 % of DM) on rumen fermentation were evaluated. In the second experiment, a single dose of 1% BSS selected from the first experiment was used to assess its effects on rumen fermentation when combined with monensin. The second experiment was conducted utilizing dual flow continuous culture fermenters.

2.1. Animals and collection of rumen fluid

Institutional Animal Care and Use Committee from the University of Minnesota approval (IACUC # 0706A09602) was obtained prior to commencement of this study. A ruminally cannulated mature cow (Montbeliarde × (Jersey × Holstein)) in early lactation served as rumen fluid donor. The cow was provided with a diet (Table 4.1) to meet or exceed the NRC requirements of a Holstein cow producing 40 kg milk/day, with 3.8% fat and 3.7% protein (NRC, 2001).

2.2. Experiment 1 - Diet, treatments and analytical procedures

A basal diet was formulated according to Table 4.2 and enriched with BSS (Sigma-Aldrich, St. Louis, MO) to attain dietary treatments with a final concentration of 0 (control), 0.5, 1, 2 and 4% of BSS dietary DM. All ingredients were ground in a Willey mill (Arthur Thomas, Philadelphia, PA) to pass through a 2-mm screen. 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; urea, 0.4.

Batch culture incubations were conducted with five replicates per treatment during two consecutive 24-h periods. At the beginning of the incubation, rumen fluid was manually obtained from the donor animal, strained through four layers of surgical gauze and transported to the laboratory in a pre-heated sealed thermos. A 2:1 McDougall's saliva:rumen fluid mix was prepared under continuous CO_2 gassing at 39.1°C and 50 mL of the mix were anaerobically transferred to 125-mL serum

bottles containing 0.5 g DM of the dietary treatments. After purging with CO_2, bottles were capped with butyl stoppers, crimp-sealed with aluminum caps and placed in an incubation bath at 39.1°C with continuous shaking at 40 oscillations/min. At 5, 10 and 24-h, volume of gas produced was measured by water displacement in an inverted burette as previously described by Kung et al. (2000). Gas sampling intervals were chosen to alleviate pressure buildup in bottles in order to minimize negative effects on fermentation (Theodorou et al., 1994). At the same sampling times, a 3-mL aliquot of gas equilibrated with atmospheric pressure was transferred from the headspace of the bottles using a gas tight syringe (Supelco, Bellefonte, PA) into a 10 mL vaccutainer tube (Tyco, Mansfield, PA) containing 5 mL of alkaline deionized water (pH = 8.5) for H_2S analysis. After 24 h, bottles were removed from incubator and decapped. Incubation fluid pH was immediately recorded (Orion 710 pH meter Thermo, Beverly, MA) and aliquots were mixed (5:1 ratio) with 25% m-phosphoric acid or 50% sulfuric acid for volatile fatty acid (VFA) and ammonia N (NH_3-N) analysis respectively.

2.3. Experiment 2 – Diets and treatments

A basal diet was formulated according to Table 4.6 and enriched with BSS (Sigma-Aldrich, St. Louis, MO) to attain dietary treatments with a final concentration of 0 (control), or 1% of BSS on a DM basis. All ingredients of the basal diet were ground through a 2-mm screen (Thomas - Willey laboratory mill Model 4, PA, USA) and resulting diets were pelleted with a CL-5 California Pellet mill

(California Pellet Mill Co., Crawfordsville, IN) to a final dimension of 6 mm in diameter x 12 mm long. Monensin sodium (MON) was purchased from Sigma Chemical Co. (St. Louis, MO). A stock solution was prepared by dissolving 100 mg of MON in 2.5 mL of ethanol and stored at -20 °C (Harford et al., 1983) for further use as described below.

2.3.1. Continuous culture operation

Eight dual flow continuous culture fermenters, described by Hannah et al. (1986) with a modified pH control and measuring system, were randomly assigned to a 2 x 2 factorial arrangement of treatments during two consecutive 10-d periods. Two levels of BSS (0 and 1% of DM) were added to the basal diet (Table 4.6) and two concentrations of MON, 0 (M0) and 5 ppm (M5), were continuously infused into the fermentation vessels via the artificial saliva. Vessels receiving M0 were provided with the same amount of ethanol that the M5 fermenters received. On day 1 of each experimental period, two fermenters were randomly assigned to each of the treatments. Fermenters were provided with 75 g of DM/d of the pelleted diet divided in eight equal portions by an automated feeding system. Each portion was provided during a 90-minute period beginning at 0300, 0600, 0900, 1200, 1800, 2100 and 2400 h daily. Liquid flow rate of fermenters was set at 8.2%/h by regulating artificial saliva input while solids dilution rate was set at 4.1%/h by regulating liquid output through filters. Artificial saliva composition was similar to experiment 1. Individual pH was recorded every 5 min by an electronic data acquisition system (Daisy Lab ®) and was maintained between 5.1 and 5.8 by automated addition of either 5N NaOH

or 3N HCl. Anaerobic conditions were maintained by continuous infusion of N_2 at a rate of 20 mL/min throughout the experiment. Fermenter temperature was maintained at 39.1 ± 0.1 °C by electrical heater.

2.3.2. Sample collection

After a 7-d adaptation period, fermenter effluents were maintained at 1°C in a water bath to retard microbial and enzymatic activities. During three consecutive days, fermenter effluents were homogenized, and three separate 500 mL aliquots were removed daily and composited by fermenter. Composite effluent samples were analyzed for total-N, NH_3-N and VFA. Freeze-dried composite samples were analyzed for DM, OM, NDF, AFD, ash, and purines. On the same days, feed port and solid outflow port of the fermenters were sealed 30 minutes prior to 0900 feeding. Nitrogen flow was adjusted to maintain 20 ± 0.1 mL/min using a digital flow meter (Aalborg GFM 17, Orangeburg, NY). After 10 minutes of stabilization, a gas sample (5 mL) from the headspace of the fermenters was taken through a septum using a gas tight syringe (Supelco, Bellefonte, PA) and transferred into a 10 mL vaccutainer tube (Tyco, Mansfield, PA) containing 5 mL of alkaline deionized water (pH = 8.5) for H_2S analysis. Hourly production of H_2S was estimated by multiplying H_2S concentration times N_2 flow for each fermenter, as previously described by Johnson et al. (2009).

2.4. Chemical analyses

Dry matter content of diets, outflows and microbial pellets was determined by drying in an oven at 105°C for 24 h. Ash was determined by weight difference after 24 h combustion at 550 °C (AOAC, 1984). At the end of each experimental period, content from each fermenter was strained through two layers of cheesecloth, centrifuged at 1,000 x *g* for 10 min to remove feed particles and the supernatant was centrifuged at 20,000 x *g* for 20 min to isolate bacterial cells. Bacterial pellets were resuspended in distilled water, frozen and lyophilized. Purine concentrations were determined by the method of Zinn and Owens (1986). Purine content of effluent and bacteria was used to partition flow of effluent N into microbial and dietary N. Total N in the effluent, bacteria, and diet was determined (AOAC, 1984). Ammonia-N was determined by steam distillation (Bremner and Keeney, 1965) using a 2300 Kjeltec Analyser Unit (Foss Tecator AB, Höganäs, Sweden). Sequential detergent fiber analyses (Van Soest et al., 1991) were used to determine NDF and ADF concentrations of the diet and effluents. Effluent VFA concentrations were determined by capillary GC analysis. Incubation fluid was solvent-extracted using ethyl acetate (3:7 ratio) during 10 minutes under continuous vortex. Samples were centrifuged at 5,000 x *g* during 5 min and supernatant was stored at -20 °C until analyzed. Analysis was performed using a Hewlett-Packard 5890 GC equipped with a Stabilwax-DA capillary column (30 m x 0.25 mm i.d., 0.25 μm film thickness, Restek, CA). Chromatographic conditions were: helium (1.7 mL/min), initial oven temperature, 110°C, held for 2.1min; ramped at 25°C/min to 200°C, held for 1.5 min;

injector temperature: 200°C, flame ionization detector temperature: 220°C; split injection (split ratio: 1/10); injection volume: 1 µL. Ethyl butyrate was included as internal standard. Hydrogen sulfide was analyzed as described by Siegel (1965), after adding to the vaccutainers 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. Samples were read at 665 nm wavelength on a Gilford Response spectrophotometer (Gilford Instrument Laboratories Inc., Oberlin, OH).

2.5. Statistical analysis

Data from experiment 1 were analyzed as a randomized complete block design by analysis of variance with Bonferroni correction. The BSS dose was considered a fixed effect and the period (block) was entered as random effect. Data from experiment 2 were analyzed as 2 x 2 factorial arrangement of treatments in a randomized complete block (period) design. Data for H_2S production and fermentation pH (obtained on 3 consecutive days) were analyzed as repeated measures with an autoregressive of order 1 structure of covariance, based on the minimum values of AIC (Akaike's Information Criterion). Lowest and highest hourly recorded pH were used for minimum and maximum pH comparison between treatments. Time (min) spent below pH 5.2, between pH 5.2 to 5.6 and above pH 5.6 was calculated using the trapezoidal integration method. Comparisons between treatments were conducted by analysis of variance. Data processing was conducted using the statistical software R (R Development Core Team, 2005). Differences were

considered significant at $P \leq 0.05$ and tendencies discussed at $0.05 < P \leq 0.1$. Unless noted otherwise, results are reported as least squares means from 4 observations/treatment.

3. RESULTS

3.1. Experiment 1

The effects of BSS on final pH and NH_3-N concentration are presented in Table 4.3. Final pH of incubations was not affected ($P > 0.05$) by addition of 0.5 or 1% BSS, compared with the control. However, an increase ($P < 0.05$) in final pH of 0.06 unit was obtained with addition of 2% BSS. Further addition of BSS (4% of DM) resulted in a larger increase ($P < 0.05$) in final pH (0.23 unit) compared with the control. Addition of 0.5% BBS resulted in an 8% reduction ($P < 0.05$) in NH_3-N compared with 0% BSS, but no changes were obtained when higher levels of BSS were supplemented.

Effects of BSS on total VFA concentration and VFA molar proportions are shown in Table 4.4. Addition of BSS up to 2% of DM had no effect ($P > 0.05$) on total VFA concentrations, but a 15% reduction ($P < 0.05$) was observed when BSS was added at 4% of DM compared with the control. Conversely, while the molar proportion of acetate remained unchanged by adding BSS up to 2% of DM, a significant increase ($P < 0.05$) was obtained when BSS was added at 4% of DM.

Molar proportion of propionate increased ($P < 0.05$) following the addition of BSS up to 2% of DM but a decrease ($P < 0.05$) was noted when BSS was added at 4% of DM. The A:P ratio decreased ($P < 0.05$) following addition of BSS at 0.5, 1 and 2% of DM, while both 0 and 4% BSS resulted in higher A:P ratios at 2.43 and 2.60, respectively. Branched-chain VFA concentration was maximized ($P < 0.05$) by 0.5% BSS compared with the control, but further addition of BSS resulted in decreasing concentrations. These variations are likely related to variations in molar proportions of isovalerate + 2-methylbutyrate because the addition of BSS did not affect ($P > 0.05$) isobutyrate proportion, averaging 2.70 mol/100 mol across treatments (Table 4.4). Absence of BSS resulted in lower ($P < 0.05$) molar proportion of valerate, compared with the other treatments.

The effect of BSS on total gas production and H_2S released is shown in Table 4.5. Addition of 0.5 or 1% BSS did not affect ($P < 0.05$) total gas production, but a 12% and 25% reduction ($P < 0.05$) was obtained when adding BSS at 2 and 4% of DM, respectively. All doses of BSS reduced ($P < 0.05$) H_2S release, with a decrease of 18, 34, 82, and 99.7% for 0.5%, 1, 2 and 4% BSS of DM, respectively.

3.2. Experiment 2

Table 4.7 shows the effect of MON and BSS on OM, NDF, ADF and NFC digestion. Addition of MON resulted in a trend ($P < 0.1$) for a decrease in apparent OM and NDF digestion at 4 and 17%, respectively, together with a 10% decrease (P

< 0.05) in ADF digestion. However, no effect ($P > 0.05$) of MON was found in OM digestion, averaging 62.5% across treatments. In contrast, addition of 1% BSS to the basal diet increased ($P < 0.05$) digestion of OM, NDF and ADF by 10, 18 and 39%, respectively compared with the control, and tended to increase ($P < 0.1$) true OM digestion. No differences ($P > 0.05$) in NFC digestion were obtained with addition of MON, but NFC digestion was 9 % lower ($P < 0.05$) when BSS was added at 1% of DM.

Effects of MON and BSS on VFA concentrations and molar proportions are summarized in Table 4.8. Addition of BSS resulted in a 27% decrease ($P < 0.05$) in total VFA concentration (73.1 to 53.3 m*M*). However, molar proportions of acetate and propionate increased ($P < 0.05$) with BSS at 9 and 5%, respectively. The larger increase in acetate proportion compared with that of propionate resulted in an increase ($P < 0.05$) in A:P ratio following addition of BSS. In contrast, addition of BSS resulted in a pronounced decrease ($P < 0.05$) in molar proportions of butyrate and caproate, with a magnitude of 35 and 55%, respectively. Molar proportions of valerate, isobutyrate and isovalerate + 2-methylbutyrate were not affected ($P > 0.05$) by addition of BSS.

Unlike observations with BSS, addition of MON did not affect ($P > 0.05$) total VFA concentration, or molar proportion of individual VFA, except for a decrease ($P < 0.05$) in molar proportion of caproate. A numerical decrease in molar proportion of acetate along with a numerical increase in molar proportion of propionate following addition of MON to the fermenters, resulted in a decrease ($P < 0.05$) in A:P ratio to

that obtained with the addition of BSS. Both treatments resulted in higher (P < 0.05) concentrations of branched-chain VFA.

Treatments effects on N metabolism are shown in Table 4.9. Ammonia-N concentration was higher than the minimum suggested by Satter and Slyter (1974) to guarantee optimal microbial growth, and similar to those reported by Kung et al. (1998) for batch culture rumen incubations. Addition of BSS resulted in a 7-fold increase (P < 0.05) in NH_3-N concentration in fermentation vessels, with a concomitant increase (P < 0.05) in daily flow of NH_3-N. An increase (P < 0.05) in dietary-N flow following the addition of BSS, along with an associated decrease in microbial-N flow, resulted in a net decrease (P < 0.05) of 5% in non NH_3-N flow from fermenters. Addition of BSS resulted in a 26% increase (P < 0.05) in dietary-N outflow from fermentation vessels. Crude protein degradation and efficiency of microbial protein synthesis (EMPS) decreased with addition of BSS at 16 and 25%, respectively.

No differences (P > 0.05) were found in NH_3-N concentration and NH_3-N outflow following addition of MON. However, outflow of non NH_3-N was 4% higher (P < 0.05) following addition of MON. This finding seems related to a numerical increase in microbial-N outflow from fermenters because no differences (P > 0.05) were obtained in the flow of dietary-N. Similarly, addition of MON resulted in no differences (P > 0.05) in CP degradation and EMPS averaging 62% and 15 g microbial N/kg OM truly digested, respectively.

Table 4.10 shows the effect of MON and BSS on H_2S production and estimated H_2S flow from continuous culture fermenters. Addition of MON resulted in a trend ($P < 0.1$) to increase H_2S concentration in the headspace of fermenters and tended ($P < 0.1$) to increase calculated H_2S flow. Addition of BSS resulted in a 98.7% reduction ($P < 0.05$) in both H_2S concentration and estimated H_2S outflow from fermenters.

Effects of MON and BSS on fermentation pH are shown in Table 4.11. Addition of MON only resulted in a trend to decrease ($P < 0.1$) total minutes spent by vessels at pH between 5.2 to 5.6, without affecting ($P > 0.05$) the mean, minimum and maximum pH of fermentations. In contrast, addition of BSS increased ($P < 0.05$) mean, minimum and maximum pH of fermentations by 0.17 pH units. As a consequence of these changes, total time spent by fermenters at pH between 5.2 to 5.6 decreased ($P < 0.05$) with a subsequent increase ($P < 0.05$) in time spent by fermenters at pH above 5.6. Similar to MON, addition of BSS did not affect ($P > 0.05$) the total time spent by fermenters at pH below 5.2.

4. DISCUSSION

4.1. Experiment 1

Bismuth subsalicylate induced small variations in final pH of short term incubations when added to the diet at 2 and 4% of DM, but not when added at lower doses. While no reports on the effect of BSS on rumen pH are available, these results

partially agree with observations from Clark et al. (1996) who noted there was not any variation in gastric pH of horses 2 h after receiving as much as 26.25 g BSS. Similarly, Dy et al. (1999) observed no change in gastric pH of human patients, 45 minutes after receiving 524 mg BSS, orally. According to Lee (1991), BSS is virtually insoluble in aqueous solutions, having no effect *per se* on pH. However, a decrease in artificial gastric fluid pH was obtained when BSS was added at a concentration of 10% wt/vol (Kappstein and Engels, 1987). In addition, Daschner et al. (1988) reported a drop in artificial gastric fluid (buffered to pH = 7), 15 minutes after addition of a 5% suspension of BSS, indicating a possible effect of dose, time, and differences between *in vitro* and *in vivo* models on resulting pH. While NH_3-N concentrations were not markedly influenced by BSS (Table 4.3), the decrease in total VFA concentration (Table 4.4) obtained with the highest dose of BSS suggests a depression in overall fermentative activity. These results agreed with the lower total gas production obtained when BSS was added to incubations at 2 and 4% of DM as shown in Table 4.5. Similar effects on VFA concentration and molar proportions were observed with other feed additives. Agarwal et al. (2009) reported an increase in A:P ratio following addition of peppermint oil to batch culture incubations. Patra et al. (2010) reported similar results when extract of clove was used, but not with extract of garlic. A dose-response increase in A:P ratio was found by Macheboeuf et al. (2008), when essential oils were added to short-term rumen incubations. According to Agarwal et al. (Agarwal et al., 2009), an increase in A:P ratio might result from an accumulation of molecular hydrogen, associated with reduced efficiency of hydrogen utilization for the synthesis of VFA. In concordance with those

observations, a reduction in total VFA concentration was obtained in the present study when A:P ratio resulted increased by the addition of BSS.

The ability of BSS to inhibit growth of pathogenic gastrointestinal bacteria has been previously established (Cornick et al., 1990; Manhart, 1990; Marshall et al., 1987). Using several strains of human pathogenic bacteria, Cornick et al. (1990) found that the 50% minimum inhibitory concentration (MIC) ranged from 512 to 4096 µg/mL, with the only exception of *Clostridium difficile* exhibiting a 50% MIC of 32 µg/mL. While these concentrations are higher than the ones used in the present study (ranging from 50 to 400 µg/mL), partial inhibition of rumen microbial population may have occured. Conversely, Suarez et al. (1998b) found that total gas production during 24-h was not affected when BSS was added at 948 µg/mL to human fecal homogenates.

In agreement with the decrease in H_2S production obtained in experiment 1 with rumen microbes, previous studies (Furne et al., 2000; Mitsui et al., 2003) have shown the effectiveness of BSS to decrease H_2S in human and rat fecal homogenates. Suarez et al. (1998b) reported a reduction of 100-fold in H_2S production at 2, 4 and 24 h following the addition of BSS at 2.6 m*M* without changes in release of dimethylsulfide, H_2, CH_4, or CO_2. However, no reduction in H_2S was obtained when BSS was added at 0.026 m*M*. Similarly, a 90% reduction in H_2S production was obtained by Mitsui et al. (2003).

In summary, addition of BSS at 1% of DM to short term rumen fluid incubations did not affect final pH, NH_3-N concentration, VFA concentration and total gas

production. Only minor changes in VFA molar proportions were obtained, but a 34% reduction in H_2S production was found. Based on these findings, addition of 1% of BSS on DM basis was chosen as the dose to conduct further studies on the effect of BSS in rumen metabolism using dual flow continuous culture fermenters.

4.2. Experiment 2

Using continuous culture fermenters, Castillejos et al. (2006) found a decrease in DM and OM digestion when 10 ppm monensin were infused. In addition, a 12% decrease in OM digestion was noted when *in vitro* incubations of different forage:concentrate proportions were enriched with 20 ppm monensin (Goiri et al., 2009), indicating a dose-dependent effect of monensin on OM digestion. Smith et al. (2010) and Quinn et al. (2009) did not find differences in dry matter degradability in batch culture incubations when monensin was added at concentrations similar to the present experiment, which is consistent with current observations.

Similar to our findings, a detrimental effect of monensin on *in vitro* fiber digestion was reported by other authors. Castillejos et al. (2006) found reductions of 0.48 and 0.94% for NDF and ADF digestion respectively when 10 ppm monensin were added to continuous culture rumen incubations. However, larger reductions were reported by Russell and Strobel (1988) following the addition of 2.5 ppm monensin to 24-h batch culture incubations of rumen fluid containing hay as a substrate. While these effects are usually related to the susceptibility of cellulolytic

ruminococci and *Butyrivibrio fribrisolven*s to monensin (Russell and Strobel, 1989), the extent of fiber digestion may be related to the source of cellulose as previously suggested by Wallace et al. (1980).

Contrary to the effect of MON, addition of BSS resulted in increases in NDF and ADF digestion, together with a marked decrease in calculated NFC digestion. These combined effects may be responsible for the observed increase in OM digestion. In contrast, the lack of differences found on true OM digestion suggests a decrease in microbial mass following addition of BSS. While no explanation is available in the literature to support these findings, several bismuth salts have a direct inhibitory effect on colonic bacteria in humans, including *Treponema, Clostridium difficile, Bacteroides fragilis, Escherichia coli, Salmonella spp., Shigella spp., Campylobacter jejuni and Campilobacter pylori* (Leon-Barua et al., 1990). The susceptibility of rumen microbes to BSS has not been established, however, other feed additives like essential oils and inactivated yeast cultures are known to enhance fiber digestion (Benchaar et al., 2006; El-Waziry and Ibrahim, 2007). Also, the improvement found in fiber digestion may be associated with an increase in mean, minimum and maximum pH of fermentations (Table 4.11). According to Hoover (1986), a reduction in pH had a major impact on fiber digestion both *in vivo* and *in vitro*. In agreement with present results, Yang et al. (2002) found a four-fold increase in NDF and ADF digestion when pH of continuous culture fermenters was artificially increased from 5.5 to 6.0, confirming previous evidence of changes in fiber digestion associated with variations in rumen pH (Mould et al., 1983; Russell and Wilson, 1996).

The change in A:P ratio following the addition of monensin is in agreement with previous *in vitro* findings in continuous culture (García et al., 2007) and batch culture (Smith et al., 2010) incubations. The classical response to monensin includes a decrease in molar proportion of acetate and an increase in propionate (Chen and Wolin, 1979; Thornton and Owens, 1981), however only numerical ($P > 0.05$) variations were obtained in the present experiment. Similar results were previously reported by other authors (Salinas-Chavira et al., 2009; Zinn et al., 1994). In addition, the classical response of VFA to monensin was obtained *in vitro* by Quinn et al. (2009) when incubated substrate contained 0.17% S, but not with 0.42% S.

As a consequence of the increase in fiber digestion, addition of BSS resulted in an increase in the molar proportion of acetate and a decrease in the molar proportion of butyrate, with a concomitant decrease in A:P ratio. Simultaneously, a reduction in total VFA was obtained probably due to a decrease in NFC digestion. As previously mentioned, there is no available information regarding the effects of BSS on rumen microbes. However, Leon-Barua et al. (1990) found a 74% decrease in fermentation activity of human colonic bacteria when BSS was added to lactose enriched stool homogenates. A similar reduction was found by Suarez et al. (1998b) using rat fecal homogenates. Interestingly, no differences in H_2, CH_4 and CO_2 released per gram of incubated substrate were found by the authors, suggesting an overall depression in fermentation, rather than a decrease in fermentation of any specific compound in the feces.

Previous research (Chen and Russell, 1991; Schelling, 1984; Yang and Russell, 1993) showed that monensin may decrease NH_3-N concentrations *in vitro* and *in vivo* by reducing rumen hyperammonia producers. However, when a low dose (10 mg/kg DM) of monensin was added to the diet of lactating dairy cows, there were no beneficial effects on N metabolism (Broderick, 2004). In addition, an increase in NH_3-N concentrations was found by Castillejos et al. (2006) when 10 ppm monensin were added to continuous culture fermenters. Busquet et al. (2005) found no effect of monensin on various N fractions when continuous culture fermenters received 1.25 or 12.5 mg monensin/L, which is consistent with the present study. Besides an effect of monensin dose, Broderick (2004) proposed an effect of substrate, showing that monensin increased NH_3-N release from acid-hydrolyzed casein but not from enzymatically hydrolyzed casein or untreated casein.

In contrast to monensin, major changes in N metabolism were induced by BSS. Increases in NH_3-N concentration and outflow appear to be related to a decrease in N utilization, resulting in a trend for lower CP degradation associated with a lower EMPS. Similar variations were previously associated with lower utilization of dietary N (Bach et al., 2007; Stern et al., 1978). In addition, the decrease in non NH_3-N outflow may be a consequence of lower microbial N flow, given the antimicrobial properties of BSS (Dupont, 2008; Manhart, 1990).

Following addition of BSS, the decrease in H_2S production obtained during the present experiment is similar to results observed in experiment 1. Using the volume and dilution rate of fermenters, it can be calculated that a daily dose of 1% BSS on a

DM basis represents a BSS concentration of 312.5 mg/L of fermenter content/d. Similar to the present study, a 96% reduction in colonic H_2S formation was found by Suarez (1998b) when humans received 400 mg BSS orally. Assuming a colonic volume of 1.2 L/d (Camilleri, 2004), this dose represents 334 mg BSS/L of colonic content, similar to the current dose.

The effect of monensin on H_2S is controversial. Kung et al. (2000) found an increase in H_2S concentration when 5 ppm monensin were added to short term rumen fluid incubations conducted with high S diets (1.1% S on a DM basis). According to the authors, indirect inhibition of monensin on methanogenic microbes may have reduced competition with sulfate-reducing bacteria leading to higher H_2S production. Alternatively, Quinn et al. (2009) reported no effect of monensin in short term incubations of dietary substrate with 0.17% S. In addition, other factors such as differences in diets of rumen fluid donors, incubated substrate composition, and rumen fluid:buffer ratio may account for disparity in results. Dietary substrate and incubation conditions in the present experiment largely differ from that previously mentioned. However, while the present experiment partially supports the findings of Quinn et al. (2009), the trend for an increase in H_2S suggests that a role of monensin on H_2S production cannot be totally excluded.

Overall, the pH values obtained in the present experiment are in agreement with the range of ruminal pH reported by long term monitoring studies in dairy cows (Zosel et al., 2010), but remained higher than those observed by Uwituze et al. (2010) in Holstein steers receiving up to 25% distiller grains in the diet. Bismuth

subsalicylate is virtually insoluble in water and has no effect on pH (Lee, 1991), but antibacterial activity against anaerobes has been demonstrated *in vitro*. Cornick et al. (1990) reported a 90% inhibition of growth in bacteria representing normal flora from health human volunteers, receiving high BSS doses (512 to 4096 μg BSS/mL). Manhart (1990) reported *in vitro* growth inhibition of diarrhea-causing bacteria following addition of BSS at doses higher than that used in the present experiment. In addition, lower total VFA concentrations, together with higher NH_3-N concentrations found following the addition of BSS may explain the increases in mean, minimum, and maximum fermentation pH, as previously reported by Koster et al. (1996). Conversely, the lack of effect of monensin on fermentation pH is in agreement with previous *in vitro* and *in vivo* reports (Kung et al., 2000; Min et al., 2005) and may be explained by lack of differences in NH_3-N and VFA concentrations.

5. CONCLUSIONS

During the present study, monensin did not affect release of H_2S and elicited only minor changes in *in vitro* rumen fermentation. In contrast, addition of BSS resulted in a dramatic reduction in H_2S release, but showed a negative impact on digestion of NFC, total VFA concentrations and nitrogen metabolism, suggesting an overall depression of rumen fermentation. Dose-titration studies are suggested to determine the optimal dose of BSS, capable of reducing H_2S without negatively

affecting rumen metabolism. Currently, feed grade BSS is not available. Future studies should also focus on the economic feasibility of BSS addition to diets for ruminants.

Table 4.1: Ingredient composition of the diet fed to the rumen fluid donor

Ingredient	% (DM Basis)
Corn silage	32.7
Ground corn	19.8
Alfalfa hay	16.1
Fuzzy cottonseed	7.50
Soybean meal 48%	3.71
Soybean hulls	3.71
Canola meal	3.71
Soypass®	3.71
Corn distillers grain	2.77
Molasses mix	2.60
Sodium bicarbonate	0.83
Meagalac®	0.65
Blood meal	0.65
Salt	0.46
Calcium carbonate	0.37
Diamond V yeast XP®	0.19
di-calcium phosphate	0.19
Potassium carbonate	0.19
Magnesium oxide	0.09
Alimet®	0.07
Sodium selenate	0.02

Table 4.2: Ingredient and chemical composition of the basal diet used in Experiment 1

Item	
Feed composition,	% DM Basis
Ground corn	50
Distillers dry grains	40
Hay	9.75
Mineral premix[1]	0.25
Chemical composition,	
CP	17.8
NDF	20.5
ADF	11.7
S	0.44
TDN	84.0

[1]Composition (on DM basis): Fe, 3.0 g/kg; Cu, 0.3 g/kg; Mn, 2.0 g/kg; Zn, 3.0 g/kg; Co, 0.1 g/kg; I, 0.1 g/kg; Se, 0.01 g/kg and vitamin A, 500,000 IU/kg; vitamin D, 100,000 IU/kg; vitamin E 100 IU/kg.

Table 4.3: Effect of bismuth subsalicylate (BSS) on final pH and NH_3-N of batch culture incubations

	BSS[1]					
Item	0	0.5	1	2	4	SEM[2]
Final pH	6.31^{c}	6.31^{c}	6.30^{c}	6.37^{b}	6.53^{a}	0.008
NH_3-N (m*M*)	29.2^{b}	26.8^{a}	28.0^{ab}	27.8^{ab}	28.2^{ab}	0.08

[1]% of DM.

[2]Standard error of the main-effect means, n=5 replicates per treatment.

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

Table 4.4: Effect of bismuth subsalicylate (BSS) on total VFA concentration and VFA molar proportions in batch culture incubations

	BSS[1]					
Item	0	0.5	1	2	4	SEM[2]
Total VFA (m*M*)	85.4^{a}	89.9^{a}	85.2^{a}	82.9^{a}	72.6^{b}	1.75
Individual VFA (mol/100 mol)						
Acetate	54.4^{b}	54.0^{b}	54.1^{b}	54.0^{b}	55.0^{a}	0.17
Propionate	22.5^{c}	22.7^{bc}	23.1^{ab}	23.6^{a}	21.2^{d}	0.13
Butyrate	14.7^{a}	14.0^{b}	13.6^{bc}	13.3^{c}	14.8^{a}	0.13
Isobutyrate	2.55^{a}	2.81^{a}	2.77^{a}	2.65^{a}	2.70^{a}	0.14
Valerate	1.90^{b}	2.52^{a}	2.59^{a}	2.64^{a}	2.55^{a}	0.12
Caproate	0.85^{a}	0.48^{c}	0.57^{bc}	0.73^{ab}	0.74^{ab}	0.04
Isovalerate + 2-methylbutyrate	3.12^{ab}	3.57^{a}	3.28^{ab}	3.09^{b}	3.01^{b}	0.12
Branched-chain VFA (m*M*)	4.83^{bc}	5.73^{a}	5.15^{b}	4.75^{bc}	4.14^{c}	0.18
Acetate:Propionate ratio	2.42^{b}	2.38^{bc}	2.34^{cd}	2.29^{d}	2.59^{a}	0.02

[1]% of DM.

[2]Standard error of the main-effect means, n=5 replicates per treatment.

[a,b]Means in the same row with unlike superscripts differ ($P < 0.05$).

Table 4.5: Effect of bismuth subsalicylate (BSS) on total gas production and H_2S release

	BSS[1]					
Item	0	0.5	1	2	4	SEM[2]
Gas production[3]	178.8[a]	178.0[a]	175.2[a]	157.7[b]	133.9[c]	2.46
H_2S production (μg)	307.2[a]	252.8[b]	201.6[c]	54.2[d]	0.75[e]	4.84

[1]% of DM.

[2]Standard error of the main-effect means, n=5 replicates per treatment.

[3]Total gas production (mL) after 24 h of incubation.

[a,b]Means in the same row with unlike superscripts differ ($P < 0.05$).

Table 4.6: Ingredient and chemical composition of the basal diet used in Experiment 2

Item	
Feed composition,	% of DM
Corn	41
Distiller dry grains	46
Hay	8
Calcium carbonate	2.5
Molasses	1.5
Trace mineral premix[1]	1.0
Chemical composition,	
CP	17.8
NDF	20.5
ADF	11.7
S	0.44
TDN	84.0

[1]Composition (on a DM basis): Fe, 3.0 g/kg; Cu, 0.3 g/kg; Mn, 2.0 g/kg; Zn, 3.0 g/kg; Co, 0.1 g/kg; I, 0.1 g/kg; Se, 0.01 g/kg and vitamin A, 500,000 IU/kg; vitamin D, 100,000 IU/kg; vitamin E 100 IU/kg.

Table 4.7: Effect of monensin and bismuth subsalicylate on organic matter and ingredient digestion

Item	MON[1]		BSS[2]		SEM[3]	*P*-value		
	0	5	0	1		MON	BSS	MON x BSS
OMD, apparent	50.5	48.3	47.0	51.9	1.11	0.08	0.002	0.07
OMD, true[4]	63.3	61.6	59.0	65.8	3.66	0.64	0.09	0.28
NFC[5]	74.3	75.2	77.6	70.9	0.73	0.23	< 0.001	0.49
NDF	43.5	36.1	27.8	32.8	2.4	0.07	< 0.001	0.45
ADF	57.7	51.7	45.6	63.7	2.6	0.04	< 0.001	0.35

[1]MON: monensin (ppm).

[2]BSS: bismuth subsalicylate (% of DM).

[3]Standard error of the mean.

[4]Corrected for contribution of bacterial OM.

[5]Non fibrous carbohydrates (calculated as 1 – CP + NDF + FAT + ASH).

Table 4.8: Effect of monensin and bismuth subsalicylate on VFA concentration in continuous culture fermenters

	MON[1]		BSS[2]			*P*-value		
Item	0	5	0	1	SEM[3]	MON	BSS	MON x BSS
Total VFA (m*M*)	67.5	61.9	73.1	53.3	2.85	0.19	< 0.01	0.86
Individual VFA (mol/100 mol)								
Acetate	53.8	52.1	50.3	54.8	1.32	0.71	0.02	0.25
Propionate	32.5	33.0	32.0	33.6	0.77	0.46	0.02	0.06
Butyrate	10.6	9.9	13.9	9.0	0.83	0.47	< 0.01	0.07
Isobutyrate	0.34	0.33	0.34	0.32	0.05	0.97	0.72	0.65
Isovalerate + 2-methylbutyrate	0.37	0.46	0.41	0.42	0.12	0.46	0.91	0.24
Valerate	0.92	0.97	1.09	0.80	0.11	0.54	0.62	0.23
Caproate	1.82	1.73	2.45	1.10	0.20	< 0.01	< 0.01	0.64
Branched-chain VFA (m*M*)	1.05	1.28	0.99	1.41	0.16	0.03	0.05	0.77
A:P Ratio	1.66	1.58	1.57	1.63	0.04	< 0.01	< 0.01	0.34

[1]MON: monensin (ppm).

[2]BSS: bismuth subsalicylate (% of DM).

[3]Standard error of the mean.

Table 4.9: Effect of monensin and bismuth subsalicylate on nitrogen metabolism in continuous culture fermenters

	MON[1]		BSS[2]			*P*-value		
Item	0	5	0	1	SEM[3]	MON	BSS	MON x BSS
NH_3-N (mg/dL)	7.41	8.74	2.04	14.11	0.88	0.14	< 0.01	0.79
N flow (g/d)								
NH_3-N	0.46	0.54	0.12	0.87	0.10	0.20	< 0.01	0.74
Non NH_3-N	7.22	7.54	7.59	7.18	0.10	0.05	0.02	0.34
Microbial-N	4.62	5.03	5.32	4.33	0.21	0.29	0.02	0.49
Dietary-N	2.60	2.52	2.26	2.85	0.18	0.49	0.06	0.24
CP degradation (%)	61.1	62.4	66.2	55.3	3.28	0.79	0.09	0.22
EMPS[4]	14.3	16.0	17.3	12.9	0.76	0.13	< 0.01	0.15

[1]MON: monensin (ppm).

[2]BSS: bismuth subsalicylate (% of DM).

[3]Standard error of the mean.

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

Table 4.10: Effect of monensin and bismuth subsalicylate on H_2S production in continuous culture fermenters

	MON[1]		BSS[2]			*P*-value		
Item	**0**	**5**	**0**	**1**	**SEM[3]**	**MON**	**BSS**	**MON x BSS**
H_2S (µg/dL)	4.35	5.45	9.67	0.13	0.52	0.08	< 0.001	0.12
H_2S flow (µg/h)	102.9	127.7	129.3	1.52	6.88	0.08	< 0.001	0.13

[1]MON: monensin (ppm).

[2]BSS: bismuth subsalicylate (% of DM).

[3]Standard error of the mean.

Table 4.11: Effect of monensin and bismuth subsalicylate on fermentation pH in continuous culture fermenters

	MON[1]		BSS[2]		SEM[3]	*P*-value		
pH	**0**	**5**	**0**	**1**		**MON**	**BSS**	**MON x BSS**
Mean pH[4]	5.41	5.38	5.31	5.48	0.05	0.64	0.01	0.28
Minimum pH[4]	5.34	5.33	5.25	5.42	0.04	0.81	0.004	0.26
Maximum pH[4]	5.47	5.43	5.37	5.53	0.04	0.55	0.006	0.15
Time below pH 5.2[5]	524.6	1082.2	889.5	717.5	386.6	0.15	0.64	0.50
Time at $5.2 \leq pH \leq 5.6$[5]	3177.4	2329.5	3275.4	2231.4	514.9	0.07	0.04	0.49
Time above pH 5.6[5]	613.1	903.4	150.0	1366.4	273.0	0.47	0.01	0.19

[1]MON: monensin (ppm).

[2]BSS: bismuth subsalicylate (% of DM).

[3]Standard error of the mean.

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

[5]Total minutes during 3 consecutive days.

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