Effects of dietary roughage and sulfur in diets containing corn dried distillers grains with solubles on hydrogen sulfide production and rumen fermentation by rumen microbes in continuous and batch culture

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Dedication

This thesis is dedicated to my husband Joe for always sticking by me.

Abstract

Dried distillers grains with solubles (DDGS) is an inexpensive feed alternative to corn. Previously, over-inclusion of DDGS has produced toxic concentrations of ruminal hydrogen sulfide (H_2S) gas, resulting in polioencephalomalacia (PEM), a degenerative brain disease. Production of ruminal H_2S requires an acidic environment conducive to converting free sulfur to H_2S in the rumen. Therefore, it was hypothesized that creating a less acidic rumen environment would help mitigate ruminal H_2S production. Two experiments were conducted to determine the effects of dietary roughage and sulfur on *in vitro* fermentation with ruminal microbes in continuous culture and batch culture. Six dietary treatments were formulated that paired 3 concentrations of sulfur (0.3, 0.4 and 0.5% of diet DM) with 2 concentrations of roughage (3 and 9% of diet DM) and are as follows: low roughage low sulfur (LRLS), low roughage moderate sulfur (LRMS), low roughage high sulfur (LRHS), moderate roughage low sulfur (MRLS), moderate roughage moderate sulfur (MRMS) and moderate roughage high sulfur (MRHS). A diet comprised of 0% DDGS was used as the control (CON) diet. Roughage had no effect on H_2S production but it did increase fermenter pH, creating a less acidic environment. In experiment 2, an increase in dietary sulfur caused an increase in total H_2S production, but there was no direct effect of roughage on total H_2S production. Higher dietary roughage created a less acidic pH but at the expense of *in vitro* fermentation, because of the lower total VFA concentration. Further investigation is needed to determine more effective methods of mitigating H_2S production using dietary manipulation, such as higher inclusion of dietary roughage or use of different roughage sources.

Keywords: continuous culture, batch culture, rumen, roughage, sulfur, hydrogen sulfide, polioencephalomalacia, dried distillers grains with solubles, feed alternative

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LITERATURE REVIEW

INTRODUCTION

Production and use of alternative fuels has become a growing industry in the United States and has led to a rise in utilization of by-product feeds by the animal industry. One example is the process of manufacturing corn ethanol, which creates many by-products used in animal diets, such as beef, swine and dairy (Thong et al., 1978; Schingoethe et al., 2009; Uwituze et al., 2011). There are two manufacturing processes for corn ethanol, wet and dry mill. Depending on the process used to create corn ethanol, results yield different by-products that can and have been used in animal diets. Much research has been focused on safety and efficacy of including these by-products as feed for cattle (Quinn et al., 2009; May et al., 2010; Neville et al., 2010; Smith et al., 2010), which is why this is an important and growing interest for animal agriculture. Therefore, it is important to understand how these by-products are derived.

PROCESSING OF CORN CO-PRODUCTS

Wet milling

This process begins with steeping of corn in large tanks of dilute sulfuric acid to soften the kernel and allow for easier extraction of corn components. During the steeping process, some nutrients are solubilized into water. The germ is extracted and used to produce corn oil while the remainder of the corn is ground and screened to remove the bran. Centrifugal force is used to isolate gluten, leaving only starch behind, which is

processed into sweeteners. Therefore, at the end of the wet milling process, there are four co-products isolated. Products include steepwater, germ, bran and gluten. Gluten is used to make corn gluten meal, a co-product high in crude protein. Bran, germ and steepwater are combined to create corn gluten feed. Germ is used in making corn germ meal, and evaporated steepwater is used to make condensed fermented corn extractives. After all components have been separated, fermentation begins to manufacture ethanol (Corn wet-milled feed products, 1982).

Dry milling

This process begins by grinding the whole corn kernel. The ground corn is sent through several cooking stages, fermentation and distillation to yield ethanol. During this time, other products are also made, such as wet distillers grains with solubles (WDGS), dried distillers grains with solubles (DDGS) and thin stillage. After fermentation and distillation steps, a product referred to as "stillage" is derived. This product is sent to a centrifuge to separate thin stillage from wet distillers grains. Wet distillers grains can also be sent through a drying system to produce dried distillers grains. Thin stillage is further dried to produce corn distillers dried solubles. When the solubles are added to dried distillers grains, the end result is DDGS (corn wet-milled feed products, 1982).

Dried distillers grains with solubles (DDGS)

This by-product of corn ethanol production has been used as a feed ingredient in swine, beef and dairy diets. Dried distillers grains with solubles is a relatively inexpensive feed, yet it provides an excellent nutrient profile. Roughly two-thirds of the dry matter of the original corn kernel is used to produce ethanol, leaving approximately

one third of the dry matter. As a result, the nutrient content is concentrated three fold. For example, when compared with dry or cracked corn, DDGS has a higher crude protein (CP), fat, acid detergent fiber (ADF) and neutral detergent fiber (NDF) content, as well as a higher digestible energy value (Spiehs et al., 2002). Because of its nutritional content, DDGS can be used to replace both concentrates and roughages because it is high in fat, protein and fiber (Spiehs et al., 2002).

Studies by Firkins et al. (1984) and Williams et al. (2010) evaluated the use of DDGS in ruminant diets and found this by-product to be a nutritionally suitable feed. However, high sulfur content of DDGS that results from processing at the plant can have detrimental effects on animals both in terms of health and performance (Zinn et al., 1997; Gould et al., 2002; Uwituze et al., 2011). Therefore, caution needs to be exercised when determining the inclusion rate at which DDGS is introduced into the diet. Addition of too much DDGS or addition too quickly can result in toxic effects from high sulfur concentrations in the diet (Gould, 2002). While sulfur is an important element in an animal's diet, its toxicity can be devastating. However, it is also important to understand the role sulfur has in the animal and that a deficiency of sulfur can be just as dangerous as toxicity.

IMPORTANCE OF SULFUR FOR RUMINANTS

Sulfur is an important mineral in ruminant diets for various reasons. It is necessary in animal diets to synthesize sulfur-containing amino acids, methionine, cysteine, homocysteine, and taurine, and the B-complex vitamins, thiamine and biotin (NRC, 2001). Biotin acts as a coenzyme for catabolism and synthesis of carbohydrates,

fats and proteins (McDowell, 1989). The classic symptom of biotin deficiency is hindquarter paralysis. Thiamine is required as a coenzyme for the metabolism of carbohydrates, fat and protein, and a deficiency can result in ataxia, loss of appetite and weight and weakness (McDowell, 1989).

Sulfur is essential for growth of rumen microorganisms and has been shown to increase cellulose, OM and ADF digestibility, especially in diets where sulfur is limited (Martin et al., 1964; Barton et al., 1971; Kennedy et al., 1971). Sulfur is necessary for cellulose digestion, and research has shown a threefold increase in cellulose digestion when sulfur was added to a diet without sulfur (Patterson et al., 1988). According to the NRC (2000), the sulfur requirement for growing and finishing cattle, gestating and lactating beef cows is 0.15% of diet DM. In dairy cattle, the requirement set by the NRC (2001) is 0.20% of diet DM. However, a large amount of sulfur is lost to rumen microbes that use sulfur to produce H_2S, which can alter requirements (Kennedy et al., 1971; Doyle et al., 1979).

Rumen microbes utilize dietary sulfur by one of two pathways: dissimilatory and assimilatory. The key difference between the two pathways is that the dissimilatory pathway releases free sulfide into the rumen whereas the assimilatory pathway does not. McDonald and Wilbur (1974) indicated that the requirement for sulfur is 20 mg of sulfate-sulfur/day and a diet containing 5 mg sulfate-sulfur/day or less is considered to be a diet deficient in sulfur. However, research has shown that as much as 40% of dietary sulfur is lost to rumen microbes (Kennedy et al., 1971; Doyle et al., 1979). Therefore it

has been suggested that 0.16 to 0.19 % of digestible OM is a more accurate guideline to determine sulfur requirements.

Sulfur metabolism in ruminants

Sulfur metabolism in ruminant animals occurs ruminally and postruminally. Sulfur in the rumen is reduced to H_2S and then converted into microbial protein, and any excess H_2S is absorbed. Postruminal metabolism of sulfur includes digestion and absorption of sulfur-containing substances, such as protein, amino acids and sulfates. Inorganic sulfate can be reduced to H_2S by dissimilatory bacteria in the rumen. Thiosulfate and sulfite can also be used to produce sulfide (McDonald and Wilbur, 1974).

Sulfur recycling can decrease the requirement of sulfur for the animal. In the blood and liver, sulfide is converted to sulfate where it is incorporated into extracellular fluid. Sulfate is recycled directly to the rumen by salivary secretion. A strong correlation has been observed between mucid salivary sulfate and blood sulfate, suggesting that the amount of sulfate in the blood parallels sulfate found in mucid salivary secretions. Once in the rumen, recycled sulfate is reduced to sulfide where it can be used to synthesize protein. Sulfur recycling accounts for approximately 2 to 5 mg of sulfur/d/kg of BW. Sulfur is lost through the excretion of urine and feces (McDonald and Wilbur, 1974). Sulfur is also expelled as hydrogen sulfide gas via eructation.

Detrimental effects of ruminal H_2S production

Sulfur is vitally important in animal nutrition; however, toxic concentrations of sulfur can be achieved. Animals acquire sulfur in various ways, such as water, feeds like DDGS and molasses, sulfur recycling and synthesized sulfur-containing amino acids by

rumen microbes. Therefore, it is necessary to take into account all sources of sulfur consumed, recycled and synthesized by the animal. For the purposes of this review, the focus will be on dietary sources of sulfur and more specifically DDGS. While DDGS can be beneficial in animal diets, over-inclusion can lead to metabolic problems. The main issue with over-inclusion of DDGS is its sulfur content.

During processing of ethanol, sulfurous compounds are used to produce ethanol and to clean equipment (Zhang et al., 2010). As a result, sulfur leaches into DDGS causing an increase in sulfur concentration. Sulfur is converted to H_2S gas in the rumen, a normal by-product of rumen fermentation. Ruminal microorganisms reduce sulfate to sulfide, which reacts with hydrogen gas to create H_2S gas. Hydrogen sulfide can be eructated; however, some of this gas is re-inhaled into the lungs. Normally the liver detoxifies H_2S through the sulfide oxidase system, but in the case of PEM, H_2S is able to bypass the liver and go directly to the brain (Kandylis, 1984). High concentrations of sulfur in the diet can lead to toxic concentrations of H_2S gas resulting in dietary induced PEM. The term PEM means softening of the brain's gray matter, and results in symptoms such as incoordination and blindness (Gould et al., 1998).

According to the NRC, the safe tolerable limit of sulfur for beef and dairy cattle is 0.4% of diet DM (2000; 2001). However, conflicting research showed that higher sulfur concentrations by inclusion of DDGS in the diet can be achieved with little to no negative effects (Neville et al, 2010). Research demonstrated that DDGS can contain as much as 0.87% sulfur (Neville et al., 2010). In addition, this problem has stimulated an increase

in research to better understand and evaluate the factors that effect ruminal H_2S production, as well as developing ways to decrease H_2S production in the rumen.

Researchers have studied many factors that could have an effect on production of ruminal H_2S. Among them include dietary sulfur concentration, ionophores, antibiotics, 9,10-anthraquinone, thiamine, digestibility of feed ingredients, type of corn processing and pH (Kung et al., 1998; Leibovich et al., 2009; Quinn et al., 2009; Neville et al., 2010; Smith et al., 2010;). These factors will be discussed in detail throughout the remainder of the review. In addition, researchers have begun to use this information to decrease ruminal H_2S production. Research found that low pH increases production of H_2S production in the rumen (Lewis et al., 1954) due to favorable acidic environment needed for conversion of sulfate to sulfide in the rumen. The rationale behind using pH to decrease H_2S production is that pH will lower the pool of hydrogen ions available to interact with sulfur to create hydrogen sulfide gas.

Factors affecting hydrogen sulfide production

Conversion of sulfide to H_2S requires uptake of H^+ ions. The ideal environment for successful formation of H_2S requires a more acidic environment compared to the near neutral pH found in the rumen. It has been postulated that an increase in pH creates a less favorable environment for conversion of sulfur to H_2S. Research has supported this postulation, showing that the ideal pH for producing H_2S is 6.5 (Lewis et al., 1954). Reduction in fibrolytic ruminal microorganisms has been shown at pH levels below 6.2, and digestion of ruminal protein decreases below pH of 5.5 (Uwituze et al., 2011). The implication in this experiment was that methods causing an increase in pH could lead to a

greater efficiency in digestion of DDGS due to a more favorable environment for rumen microbes.

There are many dietary factors that can alter ruminal pH including diet composition, rate of passage, rate of digestion, changes in diet and frequency of feeding. Diet composition is important because it determines how much fermentable substrate is available. A component of ADF is lignin which is indigestible and therefore not available to be fermented. The two main groups that classify feed are roughage and concentrate. Concentrates contain a higher percentage of fermentable substrate compared with roughages, such as sugar, starch, cellulose and hemicellulose. A decrease in fermentable substrate in the diet leads to a decrease in volatile fatty acid (VFA) production, resulting in an increase in ruminal pH. One way to achieve this effect is to provide more roughage in the diet. Roughage in the form of hay contains a greater proportion of indigestible substrate, namely lignin, which is less fermentable. Because the end products of fermentation are VFA, less substrate provided to the animal will result in a decrease in acid production or an increase in pH. In addition, it takes longer to chew hay resulting in an increase in saliva production. The buffering capacity of saliva also works to increase ruminal pH.

Rate of passage can also affect pH because food that moves too quickly through the digestive tract will not have sufficient time to be fermented and utilized by rumen microorganisms. Likewise, feed ingredients with a lower rate of digestion coupled with a high passage rate will result in an increase in unutilized feed by rumen microorganisms.

Sudden as well as dramatic changes in diet can alter ruminal pH. Therefore, it is best to gradually introduce new diets or ingredients over time, allowing for a smooth transition. Frequency of feeding also affects pH, as periods of feeding and fasting make up the pH curve. After feeding, ruminal pH decreases, and during periods of fasting it increases.

RESEARCH MODELS FOR STUDYING RUMEN FERMENTATION

There are several models for studying rumen fermentation including *in vivo*, *in vitro* and *in situ* methods. *In vivo* systems use ruminant animals for study. *In vitro* systems use rumen fluid collected from ruminant animals to conduct fermentation studies in laboratories. *In situ*, meaning “in position”, uses ruminant animals for study but substrate remains in the rumen because it does not travel through the rest of the digestive tract. Each system has benefits and limitations and often researchers will use methods in various combinations in their studies.

In vivo

An advantage to *in vivo* studies is that the subject is a live, functioning animal. Results reflect factors that *in vitro* studies cannot, such as the physical environment of the rumen, varying rates of metabolism between animals and the dilution effect from saliva production. In addition, *in vivo* studies allow researchers to observe how changes in the rumen affect other bodily systems. However, *in vivo* studies require much time, expense, and there is less control compared with *in vitro* and *in situ* studies. No two animals are exactly alike, and what makes an animal unique only adds variation to a study and can confound results. Researchers cannot standardize rates of passage or digestion between animals. In feeding trials, sorting can cause major problems as animals pick and choose

what they eat, changing the intended composition of the ration. Animals must be carefully selected to minimize differences, which can become difficult when taking into consideration factors such as breed, size, age, stage of lactation or pregnancy. Illness may require researchers to remove animals from studies to receive medical treatment. Using live animals also poses risk to researchers working with them. In addition, some *in vivo* studies are viewed negatively by animal welfare groups and the public because animal preparation typically requires multiple cannulations in the rumen and small intestine. While *in vivo* systems are ideal because they use animals as the model, the value of *in situ* and *in vitro* studies cannot be ignored. However, the impact of surgical preparation cannot be ignored either.

In situ

In situ studies often involve animals surgically prepared with a rumen cannula and use of Dacron polyester bags to suspend substrate or feed inside the rumen. Researchers can control exactly what enters the rumen as opposed to feeding a ration that can be sorted by the animal. In addition, researchers control exactly how long substrate remains in the rumen. Using this procedure, rate of digestion and degradable and undegradable nutrients can be calculated with greater accuracy. However, this information only provides a snapshot of what is occurring inside the rumen and not the entire digestive system. Time points that are chosen for incubation in the rumen may not reflect actual time spent in the rumen. However, this method can yield valuable information quickly and with minimal expense.

In vitro

In vitro study is ideal for providing maximum control and limited animal use. Often *in vitro* studies provide an initial step where researchers are able to test ideas in the laboratory before using animals. This is beneficial especially when evaluating a new additive to determine a safe and effective concentration and to extrapolate any possible negative effects. In addition, *in vitro* studies are usually much less expensive than *in vivo* studies, take less time and are more convenient for researchers. A major disadvantage to *in vitro* studies however is that the results cannot be directly applied to animal models. The results give an indication of what could be expected; however, the same results may not be obtained when using animals.

When studying rumen fermentation, there are a number of *in vitro* procedures that could be utilized. Batch culture incubations can provide a similar environment to that found in the rumen. Batch culture provides an anaerobic environment with a typical incubation of 24 to 48 hours, meaning that researchers can accumulate information in a short period of time. However, this system does not account for dilution effects of saliva, mixing effects from contractions within the rumen, absorption of nutrients through the rumen wall or how the rumen adapts to changes. However, batch culture does allow for much greater control. Because there is no absorption of nutrients, total accumulations can be measured. Rate of passage is taken out of the equation because the substrate being administered stays inside the bottle compared with an animal where there are varying rates at which food passes through the rumen. Passage can vary within animals and between animals. In summary, scientists are able to create and control an environment

where all factors are equal except what is being studied (i.e. a new feed additive, increasing concentrations of a product, etc.).

A second type of *in vitro* study is continuous culture fermentation, a vastly more complex model than batch cultures, with greater control over variables that mimic conditions in the rumen. The system includes mechanisms to control saliva flow rates into the system and flow rate out, pH, temperature, feeding intervals and anaerobic conditions. In addition, samples can be taken on a selected schedule. Gas samples, liquid and solid outflows can be taken in this system and digestibilities can be calculated. Similar to batch culture studies, continuous culture fermenters do not account for absorption of nutrients through the rumen wall. The physical environment is not the same as the rumen because there is no rumen mat, and microbial species may differ between *in vitro* and *in vivo* rumen environments.

REVIEW OF PAST RESEARCH

SAFE CONCENTRATIONS OF DIETARY SULFUR

NRC guidelines (2000; 2001) indicate that the maximum tolerable concentration of sulfur in the diet is 0.4% of dietary DM and 0.3% in high concentrate diets. However, research demonstrated that greater concentrations of sulfur can be fed to animals without negative effects (Neville et al., 2010). Lambs were fed as much as 0.87% dietary sulfur without a single case of dietary-induced PEM during the study. Lambs were initially fed a diet free of DDGS followed by a gradual increase in amounts of DDGS over a four-week period. At the end of the transition period, lambs were fed diets containing either 0.73 or 0.87% dietary sulfur, depending on treatment group. Evaluation of dietary

roughage and sulfur in beef cattle feedlot finishing diets containing DDGS demonstrated no effects on carcass characteristics (Huber et al., 2012).

The rumen requires time to adapt to changes in diet. Dietary-induced PEM is just one concern with feeding high concentrations of dietary sulfur. High concentrations of carbohydrates in an animal's diet, which are found in DDGS, can cause other metabolic problems such as acidosis in cattle. When feeding DDGS for the first time, there are two factors working to lower ruminal pH. The high concentrations of fermentable substrate found in DDGS and alteration of the diet can both decrease ruminal pH. Carbohydrates provide substrate needed for rumen microorganisms to function and survive. In return, microbes provide cattle energy in the form of VFA. As the name implies, VFA can cause the rumen environment to become acidic if there is enough substrate provided to the animal. This acidic environment is not favorable for rumen microorganisms, leading to a change in the microbial population and alteration of rumen fermentation. Clinical signs of acidosis include loss of appetite and reduced milk yield for mild cases. Serious cases of acidosis result in anorexia, sudden drop in milk production, muscular tremors, and colic symptoms (Tsuda et al., 1991). In the case of DDGS, a safe step-up program in addition to appropriate sulfur content is required to ensure proper health and well-being of the animal.

EFFECTS OF DDGS ON RUMEN FERMENTATION

In vitro dry matter disappearance

In vitro dry matter disappearance (IVDMD) can provide information regarding extent of fermentation. Feed is utilized by rumen microorganisms to produce energy.

Inclusion, dietary concentration and type of WDGS have been shown to affect IVDMD. In a study conducted by May et al. (2010), a decrease in IVDMD was observed when including DDGS in the diet compared with a control diet without DDGS (64.8 *vs.* 70.3%, respectively). They also found a negative relationship between dietary concentration of WDGS and IVDMD. An increase in WDGS from 15 to 30% resulted in a decrease in IVDMD. With corn WDGS, IVDMD decreased from 67.4 to 64.6% and sorghum WDGS decreased from 64.3 to 62.9%. Authors noted that this was due to less fermentable substrate available compared with steam-flaked corn. Smith et al. (2010) further supports this observation by showing sulfur from WDGS had no effect on IVDMD.

Type of DDGS has also been shown to affect IVDMD. Corn WDGS had a higher IVDMD compared with sorghum WDGS (May et al., 2010). Acid detergent fiber content of corn WDGS was lower than sorghum WDGS, which could explain the greater IVDMD in corn WDGS because ADF contains lignin which cannot be digested by rumen microorganisms. Because ADF content of sorghum WDGS was higher, there is less fermentable substrate available, resulting in a decrease in fermentation and IVDMD. In addition to type of DDGS, pH can also affect IVDMD.

Uwituze et al. (2008) conducted a study comparing three pH levels (5, 5.5 and 6) during an incubation period of 48 h. There was a decrease in IVDMD consistent with a decrease in pH. A decrease in pH can create a less suitable environment for certain rumen microorganisms to grow and function. Alteration in the rumen microbial population could also contribute to the decrease in IVDMD because a smaller population

demands less fermentable substrate. Leibovich et al. (2009) found that steam-flaked corn (SFC)-based diets had higher IVDMD compared with dry-rolled corn (DRC)-based diets, which is in agreement with Zinn et al. (2002). The process of steam-flaking increases starch and protein digestibility, leading to an increase in IVDMD. In addition, inclusion of 15% sorghum WDGS compared with 0% WDGS decreased IVDMD possibly due to an increase in concentration of ADF (13.5 *vs.* 10.1%, respectively). Research has shown no effect of sulfur on IVDMD in batch culture incubations (Quinn et al., 2009; Smith et al., 2010).

In vitro gas production

Gas production is an indication of the extent of fermentation in the rumen because it is a by-product of fermentation. In general, a decrease in gas production is not desirable because it indicates a compromise in rumen fermentation and digestibility. This decrease in gas production could be caused by illness or disease, a decrease in DM consumption or a change in diet. When a change in gas production is caused by a change in diet, there are several possible explanations. The new diet may be less digestible because of a higher fiber content that is not readily available for rumen microorganisms. Another possibility is that an additive has been included in the diet that depresses functionality of rumen microorganisms, perhaps killing some of the microbial population. Current research has explored these possible causes, focusing specifically on inclusion of distillers grains, dietary sulfur and corn processing methods.

Inclusion of DDGS negatively affected total gas production in studies by May et al. (2010) and Leibovich et al. (2009). In the latter study, diets containing DDGS

compared to those without DDGS resulted in a decrease in total gas production (208.7 *vs.* 189.8 μL, respectively). They demonstrated that as WDGS inclusion went from 15 to 30%, there was a decrease in total gas production, likely due to an increase in fiber concentration when including WDGS and increasing its concentration. An increase in fiber content provides less fermentable substrate for rumen microorganism, resulting in a decrease in VFA production and its gaseous by-products. Because DDGS can be a major source of sulfur in the diet, research has been conducted to evaluate its effect on gas production.

Effects of sulfur on total gas production have been conflicting (Quinn et al., 2009; Smith et al., 2010). Quinn et al. (2009) observed a tendency for total gas production to increase over a 24-h incubation for cultures with supplemental dietary sulfur (0.42% of DM). They concluded that this might be evidence that very high concentrations of sulfur can influence rumen fermentation. However, other studies observed that sulfur (0.14% of DM) had no effect on total gas production (Smith et al., 2010). It should be noted that there was an approximately three-fold difference in dietary sulfur content between the two studies, which could explain conflicting results and perhaps suggest that a minimal requirement of dietary sulfur is needed to affect total gas production.

Corn processing method has also been studied for its effect on gas production. Leibovich et al. (2009) detected an increase in total gas production for SFC-based diets compared with DRC-based diets (210.3 *vs.* 188.1 μL, respectively). As mentioned previously, steam-flaking of corn increases digestibility of starch and protein, resulting in a greater amount of fermentable substrate compared with dry rolling corn. An increase in

fermentable substrate would lead to an increase in gaseous by-products resulting from fermentation in the rumen.

Volatile fatty acids

Volatile fatty acids are produced when rumen microorganisms convert feed into energy. Increases and decreases in VFA production can provide an indication of rumen microbial efficiency. Because this process requires fermentation of feed, a decrease in VFA production can indicate health status, a lower digestibility of feed or perhaps a decrease in DM intake. Sulfur has shown no major effect on VFA production or concentration (Kung et al., 1998; May et al., 2010; Smith et al., 2010). May et al. (2010) also showed no difference in VFA production or concentration between corn and sorghum WDGS or concentration, 15 *vs.* 30% of diet DM. In addition, Smith et al. (2010) noted that an increase in sulfur concentration in the diet had no effect on the acetate:propionate (A:P) ratio.

FACTORS AFFECTING H_2S PRODUCTION

Increases in dietary sulfur have resulted in an increase in H_2S production (Quinn et al., 2009; May et al.2010; Smith et al., 2010). Type of DDGS has also been evaluated to determine its effect on H_2S production. In a study conducted by May et al. (2010), corn and sorghum WDGS were used in a 24-h batch culture incubation at 15 and 30% of diet DM. Both sources of WDGS had sulfur contents of 0.2 and 0.3% for the 15 and 30% WDGS diets, respectively. There was no difference in the amount of H_2S produced between corn and sorghum WDGS. Hydrogen sulfide production increased with higher

DDGS concentrations, demonstrating that the amount of sulfur, not the type of WDGS, affected H_2S production.

Leibovich et al. (2009) evaluated the effect of corn processing on H_2S production in diets containing sorghum WDGS. Steam-flaked corn and DRC-based diets were evaluated. Hydrogen sulfide production was similar for SFC and DRC-based diets. There was no difference in H_2S production between 0 and 15% sorghum WDGS diets, which is possibly due to similar sulfur concentrations between dietary treatments (0.20, 0.24, 0.23, and 0.21 for the DRC-based, 0% sorghum WDGS; DRC-based, 15% sorghum WDGS; SFC-based, 0% sorghum WDGS; and SFC-based, 15% SWDGS diets, respectively).

Dietary sulfur and mineral interactions have been shown to impact H_2S production in the rumen. Molybdenum has inhibited and stimulated H_2S production. In sheep, administration of 48 mg Mo/kg of feed increased ruminal sulfide concentrations, however, 76 mg Mo/kg of feed decreased ruminal sulfide concentrations. Copper also affects sulfide production by reducing the effects of Mo, therefore Cu has an effect on ruminal sulfide production (Tsuda et al., 1991).

REDUCING RUMINAL H_2S PRODUCTION

Research has been conducted in an attempt to decrease ruminal H_2S production. Substrates have been added to the diet such as 9,10-anthraquinone (AQ) and bismuth subsalicylate (BSS) in addition to vitamins and minerals. Kung et al. (1998) used 24-h batch culture to examine a high sulfur diet containing 1.09% sulfur of diet DM supplemented with either 1, 10 or 25 ppm of AQ. In comparison with the high sulfur diet

that contained no AQ, there was a reduction in H_2S production of 9, 71 and 89% with 1, 10 or 25 ppm of AQ, respectively. Based on *in vitro* data, addition of AQ has potential to reduce H_2S production in the rumen. In a study conducted by Ruiz-Moreno (2012), BSS was used in a 24-h batch culture incubation to determine the effect on H_2S production. Concentrations of BSS used were 0.5, 1, 2 and 4% of the diet DM. Compared with the control containing no BSS, H_2S concentrations were reduced by 18, 24, 82 and 99%, respectively. However, rumen fermentation was greatly suppressed with high BSS inclusion. When BSS was supplemented at 4%, there was a decrease in VFA concentration by 15% and a subsequent decrease in pH of 0.22 units.

In addition to these substrates, vitamins and mineral have been used to decrease ruminal production of H_2S. It is unclear what role thiamine plays in PEM or how it interacts with S; however, research has indicated that using thiamine may decrease H_2S production (Brent and Bartley, 1984; McDowell, 1989; Olkowski et al., 1992). Neville et al. (2010) assessed the effect of thiamine concentration on ruminal H_2S concentration and found that thiamine inclusion affected H_2S concentration, resulting in an increase in H_2S when comparing lambs fed 150 mg thiamine per animal per day to those fed 50 mg/d. They also showed that supplementing thiamine in a high (0.87%) sulfur diet resulted in a decrease in H_2S concentration compared with animals fed a high (0.71%) sulfur diet without thiamine supplementation. The authors note that more research is required to further understand the effect of sulfur source in feed and water and metabolism of sulfur in ruminant animals. Molybdenum has also been used to decrease H_2S production. In a study conducted by Kung et al. (2000), they evaluated effects of 1, 10 and 25 ppm of

molybdenum with high sulfur diets (109 g/kg) in *in vitro* batch culture incubations. There was a decrease in H_2S production of 12 and 77% for 10 and 25 ppm treatments, respectively. In addition, there was no effect of molybdenum on batch culture pH, ammonia-N, methane and hydrogen gas production.

CONCLUSIONS

Research has demonstrated the importance of sulfur in the ruminant diet but also the detrimental effects of its toxicity. It is critical that more research be conducted to further understand and improve the safe use of DDGS in ruminant diets. A low ruminal pH has been shown to be favorable for production of H_2S (Lewis et al., 1954). It is therefore hypothesized that manipulation of ruminal pH to create an unfavorable environment for production of H_2S may be a viable solution. These reasons justify further studies to examine effects of dietary sulfur and roughage in ruminant diets containing DDGS.

Effects of dietary roughage and sulfur in diets containing corn dried distillers grains with solubles on hydrogen sulfide production and rumen fermentation by rumen microbes in batch and continuous culture

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Abstract. Dried distillers grains with solubles (DDGS) have been used in production animal diets. Overuse of DDGS can cause toxic concentrations of ruminal hydrogen sulfide gas (H_2S), resulting in polioencephalomalacia, a deleterious brain disease. Because H_2S gas requires an acidic rumen environment and diet can influence ruminal pH, it has been postulated that dietary manipulation could help mitigate H_2S production. Therefore, the objective of this study was to assess the effect of dietary roughage and sulfur concentrations on H_2S production and rumen fermentation. In experiment 1, 7 dual-flow continuous culture fermenters were used in 4 consecutive 9-d periods consisting of 6 d of adaptation followed by 3 d of sampling. In experiment 2, at the conclusion of each 9-d continuous culture period, adapted rumen fluid was used for

inoculation of 24-h batch culture incubations. In both experiments, 6 dietary treatments were formulated to consist of 0.3, 0.4 or 0.5% dietary sulfur and 3 or 9% dietary roughage, using grass hay as the roughage source. A corn-based diet without DDGS was used as a control diet. Headspace gas was sampled to determine H_2S production and concentration. In experiment 1, higher dietary roughage had no effect on H_2S production, but did create a less acidic environment due to an increase in *in vitro* pH. In experiment 2, an increase in dietary sulfur caused an increase in ruminal H_2S production, but there was no direct effect of dietary roughage on H_2S production. Higher dietary roughage resulted in a less acidic batch culture pH but reduced total VFA concentration. Further investigation is needed to determine a more effective way to mitigate ruminal H_2S production using dietary manipulation, which could include higher inclusion of dietary roughage or the use of different roughage sources.

Keywords: continuous culture, batch culture, rumen, roughage, sulfur, hydrogen sulfide, polioencephalomalacia, dried distillers grains with solubles, alternative feed

INTRODUCTION

When corn is used for ethanol production, there is less corn available to feed livestock. However, there are several by-products available after corn ethanol has been produced that are suitable for livestock diets. Of particular interest is the use of dried distillers grains with solubles (DDGS), which is a high quality feed. After corn ethanol has been produced, there is an approximate three-fold increase in nutrients in DDGS (Spiehs, et al., 2002), and contains approximately 30% CP, 45% NDF and 11% crude fat. Digestible and metabolizable energy of DDGS was calculated at 3.99 and 3.75 Mcal/kg,

respectively (Spiehs et al., 2002). Rumen undegradable protein (RUP) accounts for approximately 55% of CP (Grings et al, 1992) in DDGS.

Inclusion of DDGS has been implemented in various animal diets including beef cattle, dairy cattle and swine (Thong et al., 1978; Schingoethe et al., 2009; Uwituze et al., 2011). However, a disadvantage to using DDGS in animal diets, particularly in ruminants, is the high sulfur content found in distillers grains. These high concentrations of sulfur are present due to the use of sulfuric acid in cleaning pipes in corn ethanol plants. In spite of this observation, sulfuric acid is still used to regulate pH during fermentation of corn to produce ethanol.

In cattle, high dietary concentrations of sulfur in DDGS can cause a toxic increase in ruminal H_2S production and dietary-induced polioencephalomalacia (PEM) (Gould et al., 1991; Gould et al., 1997). Symptoms of PEM include blindness, incoordination and sometimes recumbency with seizures (Gould, 1998). The NRC for beef cattle (2000) and dairy cattle (2001) recommended that in general the maximum tolerable limit of sulfur in diets fed to cattle is 0.4 or 0.3% in high-concentrate diets.

Research has been conducted to develop new ways to reduce ruminal hydrogen sulfide production (Kung et al., 1998; Kung et al., 2000; Quinn et al., 2009; Smith et al., 2010). However, much of that research has focused on using additives to decrease production of hydrogen sulfide (H_2S) in the rumen (Kung et al., 1998; Kung et al., 2000; Smith et al., 2010). Lewis et al. (1954) showed that pH can have a significant effect on conversion of sulfate to H_2S gas. Producing H_2S gas requires an acidic environment for optimal production with a pH around 6.5 (Lewis et al., 1954). However, lower pH (~5.5)

has been implicated to be optimal for hydrogen gas (H_2) production from glucose (Fang et al., 2002). Hydrogen gas is also an important H-donor for producing H_2S (Lewis et al., 1954). In addition, lower pH creates an even greater H-donor pool.

From this information it is hypothesized that an increase in *in vitro* pH can create an unfavorable environment for H_2 gas production. *In vitro* pH decreases when VFA are produced because of their acidity. Conversely, if VFA production can be lowered, it should be possible to achieve higher *in vitro* pH values. Volatile fatty acids are produced when there is ample availability of fermentable carbohydrate as substrate for ruminal microbes. Roughage sources, such as hay, contain lignin which cannot be digested at the same rate or extent as highly fermentable grain sources. This indigestible fraction lowers the amount of fermentable substrate available, thus decreasing VFA production and increasing *in vitro* pH (May et al., 2010). Therefore, the objective of the experiment was to evaluate effects of dietary roughage and sulfur on H_2S production and rumen fermentation from corn-based diets containing DDGS in continuous culture culture and batch culture incubations.

MATERIALS AND METHODS

The University of Minnesota Institutional Care and Use Committee approved all animal use in this study (IACUC Protocol:1304-30557A).

Experimental diet

All dietary ingredients (Table 1) were ground though a 2-mm screen and were sent to DairyOne (Ithaca, NY) for analysis (Table 1) of DM, CP, ADF, NDF and sulfur

content. Ingredients were mixed and pelleted (CL-5, California Pellet Mill Co., Crawfordsville, IN) to a final size of 6 and 12 mm in diameter and length, respectively, to yield 7 dietary treatments formulated to be isonitrogenous at 17.5% CP for *in vitro* continuous culture fermentation and *in vitro* batch culture studies. Pelleted diets were sent to DairyOne for analysis as described above for dietary ingredients. The same dietary treatments were used in experiments 1 and 2.

Collection of ruminal fluid

Ruminal fluid was collected from a ruminally cannulated cross-bred dairy cow housed at the University of Minnesota Dairy Teaching and Research Facility in St. Paul, MN. The donor cow was fed a 60:40 forage to concentrate diet containing corn silage (32.7% of diet DM), a lactation protein mix (21.3% of diet DM), ground corn (19.8% of diet DM), alfalfa hay (16.1% of diet DM), fuzzy cottonseed (7.5% of diet DM) and a molasses mix (2.6% of diet DM). Ruminal fluid was collected, strained though 4 layers of cheesecloth and immediately placed in a sealed thermos container for transport to the laboratory. Within approximately 30 minutes after sampling, fluid was inoculated into each fermenter.

Continuous Culture Operation

The continuous culture fermenter system was a modification of the system of Hoover et al. (1976), as described by Hannah et al. (1986). Seven dual-flow continuous culture fermenters were used in 4 consecutive periods. Each period consisted of 6 days of adaptation followed by 3 days of sample collection. Dietary treatments were assigned randomly to fermenter. Fermenters were provided with 75 g of dietary substrate DM that

was administered over 8, 1.5-hour periods. An automated feeding device (Hannah et al., 1986) controlled by a timer (DT 17, Intermatic, Spring Grove, IL) was used to regulate feeding duration and schedule. Each 1.5 h feeding period was followed by 1.5 h of rest. Artificial saliva was prepared according to Weller and Pilgrim (1974) and infused continuously into fermenters, and contained 0.4 g/L of urea to simulate nitrogen (N) recycling**.** Saliva was constantly infused into fermenters to attain an 8.2%/h liquid dilution rate to simulate passage in beef cattle. Solids dilution rates were adjusted daily to 4.1%/h. Fermenter pH was maintained at a range between 5.0 to 7.0 via computer system by automatic addition of concentrated 3 *N* HCL or 5 *N* NaOH to correct pH. Culture pH was recorded every 5 min by an electronic data acquisition system (Daisy Lab®, National Instrument Services, TX, USA). Anaerobic conditions were maintained by continuous N_2 infusion, while fermenter temperature was maintained at 39°C.

Sample collection and analytical procedures

During sampling days, collection vessels were kept in a 1°C water bath to cease microbial action. Each sampling day at 1000 h, solids and liquid effluent were combined by fermenter. Total effluent was homogenized (PT10/3S homogenizer, Kinematica GmbH, Bohemia, NY) for 2 min, and a subsample of 500 mL was taken, composited with the previous collection and kept frozen at -20°C until analysis for total N, ammonia-N (NH_3-N) and VFA. A subsample (approximately 600 mL) of the 1500 mL composited effluent from each fermenter was lyophilized and used for analysis of DM, OM, NDF, ADF, ash and purines.

Bacterial pellets were isolated from fermenters at the end of each period. Fermenter contents were strained though 2 layers of cheese cloth. Fluid was then centrifuged at 1,000 x *g* for 10 min. The supernatant was recovered and centrifuged at 20,000 x *g* for 20 min. Supernatant was discarded and the bacterial pellet recovered and lyophilized. Total N in the effluent, bacteria and diet was analyzed by the macro-kjeldahl method (AOAC, 1995). Ammonia-N was analyzed by steam distillation using a Kjeltech 2300 Analyzer unit (Tecator, Herdon, VA, USA). The ADF and NDF concentrations of diets and fermenter effluents were analyzed using the procedure of Van Soest et al. (1991). Purine concentrations for bacterial pellets and fermenter effluents were determined and used to calculate bacterial and dietary-N (Zinn and Owens, 1986).

H_2S analysis

A 3 mL subsample of headspace gas was taken at 1000 h on sampling days directly from each fermenter and injected into a 10 mL vaccutainer containing 5 mL of alkaline water (pH = 9.5 to 10.0). To determine H_2S concentrations, a colorimetric reaction was conducted. Each vaccutainer was injected with 0.5 mL of ferric chloride solution and 0.5 mL of 4-amino-*N,N* dimethylaniline sulfate solution and left to develop the reaction at 25°C for 30 min. Absorbance was determined using a spectrophotometer at 665 nm (Stasar II, Gilford, Oberlin, OH). A standard curve was used to convert absorbance to concentration.

VFA analysis

Effluent VFA concentrations were determined by capillary GC analysis. Incubation fluid was solvent-extracted using ethyl acetate (3:7 ratio) during 10 min under

continuous vortex. Samples were centrifuged at 5000 x *g* for 5 min and supernatant was stored at -20°C until analyzed. Analysis was performed using a HP 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 (1.9 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.

In vitro batch culture incubation

On the last day of sampling from the continuous culture fermenters, a 24-h batch culture incubation (experiment 2) was conducted using adapted rumen fluid. Initial pH was recorded from the computer monitoring system at the time of inoculation. Serum bottles (25 mL) containing 0.2 g of dietary DM were inoculated with 10 mL of adapted rumen fluid and 10 mL artificial saliva (3 replications, 21 total observations) and flushed with N_2. Bottles were capped with a rubber stopper, crimp-sealed and incubated in an oscillating shaking bath (Reciprocal shaking bath model 50, Precision Scientific, Chicago, IL) at 39°C for 24 h. A subsample (3 mL) of headspace gas was taken for H_2S analysis and total gas was measured at 5 and 24 h. Total gas production was taken using an inverted, water-filled burette. Gas was collected using a 5-mL gas-tight syringe. At the end of the 24-h period, final pH was recorded and two 5-mL subsamples of fluid were taken for VFA and NH_3-N analysis. Fluids were acidified with 1 mL each of 25% metaphosphoric acid and 50% sulfuric acid for VFA and NH_3-N analysis, respectively.

Statistical analyses

For both experiments, data were analyzed using the MIXED procedure in SAS (SAS Inst. Inc., Cary, NC, 2013). Data from DDGS-containing diets were analyzed as a randomized complete block design with a 3 x 2 factorial arrangement of treatments. Dietary sulfur and roughage, and their interaction was included in the model as a fixed effect, and period was a random effect. Contrasts were used to compare CON *vs* DDGS-containing diets. Satterthwaite's approximation (1941) was used to calculate the denominator degrees of freedom. All treatment results were reported as least squares means, with significance declared at $P < 0.05$, and a trend was declared at $P < 0.10$.

RESULTS

Experiment 1

Effects of roughage and sulfur on H_2S production, and digestion of OM, NDF and ADF in DDGS diets from continuous culture fermenters are shown in Table 2. Greater ($P < 0.05$) H_2S production (1.61 *vs.* 0.13 µg) and concentration (0.54 *vs.* 0.04 µg/mL) were observed for dietary treatments containing DDGS compared with CON.

Apparent OM digestion was not affected by roughage, sulfur or the interaction between roughage and sulfur ($P > 0.10$). Higher concentrations of dietary sulfur treatments tended ($P = 0.10$) to increase true OM digestion (58.3 *vs.* 45.6% for moderate and low sulfur, respectively). Inclusion of DDGS lowered ($P < 0.05$) apparent OM digestion compared with CON (26.4 *vs.* 32.4%, respectively). No difference in true OM digestion was detected with inclusion of DDGS in the diet ($P > 0.10$).

There was no effect of roughage, sulfur or interaction between roughage and sulfur on NDF digestion ($P > 0.10$), but ADF digestion decreased ($P < 0.05$) between moderate and low dietary roughage (23.1 *vs.* 35.2% for moderate and low roughage dietary treatments, respectively) and increased ($P < 0.05$) with dietary sulfur (26.5 and 24.8 *vs.* 36.1 for low, moderate and high sulfur dietary treatments, respectively). Neutral detergent fiber digestion was similar between DDGS dietary treatments and the CON diet ($P > 0.10$). No differences were detected in ADF digestion between DDGS-containing diets and CON ($P > 0.10$).

Effects of roughage and sulfur on continuous culture pH are shown in Table 3. There was an interaction (P < 0.05) between roughage and sulfur. The LRLS dietary treatment had the lowest pH (5.12) and LRMS, MRMS and MRHS diets had the highest pH (5.21, 5.21 and 5.23, respectively). The LRHS and MRLS diets had moderate pH (5.17 and 5.16, respectively). Inclusion of DDGS produced higher ($P < 0.05$) fermenter pH values compared with CON (5.18 *vs.* 5.09, respectively).

Effects of roughage and sulfur on total VFA concentration and individual VFA of DDGS dietary treatments are shown in Table 4. Total branched-chain VFA concentration and molar proportions of propionate and branched chain VFA were not affected by dietary roughage or sulfur ($P > 0.10$). Higher dietary sulfur treatments tended ($P = 0.10$) to have lower total VFA concentration compared with low and moderate sulfur dietary treatments (135.6 *vs.* 158.0 and 157.3 m*M*, respectively) with no effect of dietary roughage ($P > 0.10$). Dietary roughage had no effect on acetate:propionate (A:P; $P > 0.10$) ratio but there was a tendency ($P = 0.09$) for high dietary sulfur treatments to

have a lower A:P ratio compared with moderate sulfur dietary treatments (2.8:1 *vs.* 3.2:1, respectively). Molar proportions of acetate were negatively affected ($P < 0.05$) by high sulfur dietary treatments (54.8 *vs.* 62.5 and 60.9 mol/100 mol for high, low and moderate sulfur dietary treatments, respectively), but no effect of dietary roughage was observed ($P > 0.10$). An increase in dietary sulfur resulted in higher ($P < 0.05$) molar proportions of butyrate compared with low and moderate dietary sulfur treatments (20.0 *vs.* 14.5 and 15.4 mol/100 mol, respectively), but there was no effect of dietary roughage ($P > 0.10$).

There was no effect of DDGS inclusion on molar proportion of branched chain VFA concentration ($P > 0.10$). Addition of DDGS to the diet reduced ($P < 0.05$) total VFA concentration (149.1 *vs.* 203.7 m*M*), total branched chain VFA concentration (0.9 *vs.* 1.3 m*M*), A:P ratio (3.0:1 *vs.* 4.1:1, respectively) and molar proportions of acetate (58.7 *vs.* 68.2 mol/100 mol), but led to an increase ($P < 0.05$) in molar proportions of propionate (20.1 *vs.* 16.7 mol/100 mol) and butyrate (16.7 *vs.* 12.5 mol/100 mol) compared with CON.

Effects of roughage and sulfur on N metabolism of DDGS-containing dietary treatments are shown in Table 5. Addition of dietary sulfur increased ($P < 0.05$) NH_3-N concentration from 1.15 and 1.37 to 2.76 mg/dL for low, moderate and high sulfur dietary treatments, respectively. As dietary sulfur increased, daily NH_3-N flow (0.06 *vs.* 0.02 and 0.03 g/d for high, low and moderate dietary treatments, respectively) and efficiency of microbial protein synthesis (EMPS) (46.7 *vs.* 37.7 and 39.3 g of microbial N/kg of OM truly digested for high, low and moderate sulfur dietary treatments, respectively) increased ($P < 0.05$) and daily dietary-N flow (0.67 and 0.62 *vs.* 1.19 g/d for moderate,

high and low dietary sulfur treatments, respectively) decreased ($P < 0.05$). Low dietary roughage treatments had higher ($P < 0.05$) daily non-ammonia N (NAN) flow compared with moderate dietary roughage treatments (2.40 *vs*. 2.31 g/d, respectively). Daily microbial-N flow increased ($P = 0.05$) with higher concentrations of dietary sulfur (1.67 and 1.73 *vs.* 1.22 g/d for moderate, high and low dietary sulfur treatments, respectively). Inclusion of DDGS decreased ($P < 0.01$) in NH_3-N concentration (1.76 *vs.* 4.75 mg/dL, respectively) and daily NH_3-N flow (0.03 *vs.* 0.09 g/d, respectively) and increased ($P < 0.05$) in daily NAN flow (2.35 *vs.* 2.22 g/d, respectively). The DDGS diets tended ($P = 0.09$) to have greater EMPS (41.3 *vs.* 36.1 g of microbial N/kg of OM truly digested, respectively) compared with a CON diet.

Experiment 2

Effects of roughage and sulfur on H_2S production of DDGS treatments in batch culture fermentation are shown in Table 6. An increase ($P < 0.05$) in total H_2S production from 42.2 to 81.9 μg for low and high dietary sulfur treatments, respectively, and a tendency for an increase ($P = 0.09$) in total gas production from 35.8 and 36.0 to 37.4 mL for low, moderate and high dietary sulfur treatments was consistent with an increase in dietary sulfur. There was a tendency ($P = 0.08$) to increase final pH from 5.61 to 5.71 with low and high sulfur dietary treatments, respectively. Moderate roughage treatments had a higher ($P < 0.05$) final pH (5.71 *vs.* 5.61, respectively) and tended ($P = 0.08$) to have a greater change in pH (0.43 *vs.* 0.36, respectively) compared with low dietary roughage treatments. Inclusion of DDGS resulted in greater ($P < 0.05$) total H_2S production (64.9 *vs.* 21.2 μg, respectively) and concentration (1.77 *vs.* 0.56

μg/mL, respectively) and a reduction ($P < 0.05$) in total gas production (36.4 *vs.* 38.9 mL, respectively) compared with CON. Higher ($P < 0.05$) initial (5.26 *vs.* 5.09, respectively) and final pH (5.66 *vs.* 5.44, respectively) was observed for DDGS-containing diets. No difference was detected between DDGS-containing diets and CON for change in pH ($P > 0.10$). There was no effect of roughage, sulfur or interaction between roughage with sulfur on NH_3-N ($P > 0.10$). Inclusion of DDGS did not affect NH_3-N when compared with CON ($P > 0.10$).

Effects of roughage and sulfur on batch culture VFA concentration of DDGS-containing dietary treatments are presented in Table 7. There was no interaction between dietary roughage and sulfur on any VFA measurements ($P > 0.10$). There was no effect of sulfur on total VFA concentration ($P > 0.10$) but moderate dietary roughage treatments had lower ($P < 0.05$) total VFA concentration (119.1 *vs.* 134.7 m*M*, respectively) than low dietary roughage treatments. There was no effect of dietary roughage or sulfur on total branched-chain VFA concentration ($P > 0.10$). Dietary sulfur caused decreases ($P < 0.05$) in the A:P ratio from 2.7:1 and 2.6:1 to 2.1:1 for low, moderate and high sulfur dietary treatments, respectively. Dietary roughage tended ($P = 0.10$) to lower A:P ratio from 2.6:1 to 2.3:1 for low and moderate roughage, respectively. Dietary sulfur lowered ($P < 0.05$) the molar proportion of acetate from 58.3 and 55.9 to 46.6 mol/100 mol for low, moderate and high sulfur, respectively. There was a tendency ($P = 0.10$) for dietary roughage to also lower the molar proportion of acetate from 55.8 to 51.5 mol/100 mol for low and moderate roughage, respectively. There was no effect of dietary roughage or sulfur on the molar proportion of propionate ($P > 0.10$). There was also no effect of

dietary roughage on the molar proportion of butyrate ($P > 0.10$); however, high dietary sulfur treatments showed greater ($P < 0.05$) molar proportions of butyrate compared with low and moderate dietary sulfur treatments (20.9 *vs*. 14.3 and 15.7 mol/100 mol, respectively). High dietary sulfur treatments caused ($P < 0.05$) molar proportions of branched-chain VFA to be higher compared with low dietary sulfur treatments (1.9 *vs.* 1.3 mol/100 mol, respectively) and tended ($P = 0.06$) to be higher than moderate dietary sulfur treatments (1.9 *vs.* 1.5 mol/100 mol, respectively).

There was no effect of DDGS inclusion on total branched-chain VFA concentration and molar proportion of butyrate compared with CON ($P > 0.10$). Inclusion of DDGS lowered ($P < 0.05$) total VFA concentration (125.6 *vs.* 153.3 m*M*, respectively), A:P ratio (2.4:1 *vs.* 3.2:1, respectively) and molar proportion of acetate (53.1 *vs.* 61.8 mol/100 mol, respectively), and resulted in greater ($P < 0.05$) molar proportions of propionate (22.6 *vs.* 19.6 mol/100 mol, respectively) compared with CON. There was a tendency ($P = 0.06$) for molar proportions of branched-chain VFA to be higher with DDGS inclusion compared with CON (1.7 *vs.* 1.1 mol/100 mol, respectively).

DISCUSSION

Experiment 1

The objective of this experiment was to determine if dietary roughage could mitigate production of H_2S production caused by DDGS inclusion without causing further detrimental effects on microbial fermentation in continuous culture. Inclusion of DDGS resulted in higher H_2S production and concentration compared with CON, which

is in agreement with previous studies (May et al., 2010; Leibovich et al., 2009) and is due to an increase in dietary sulfur. The interaction effect between dietary roughage and sulfur on continuous culture pH suggests that there may be a possible slight buffering effect of roughage on low and high sulfur dietary treatments. The MRLS had higher pH than LRLS (5.16 vs. 5.12, respectively) and MRHS had higher pH than LRHS (5.23 vs. 5.17, respectively).

There was no effect of the interaction of dietary roughage or sulfur on ADF digestion. However, reducing dietary roughage concentration from 9 to 3% resulted in higher ADF digestion (23.1 *vs*. 35.2%, respectively). Fibrolytic bacteria do not function well under acidic environmental conditions. Therefore, optimal fiber digestion will occur when pH is higher, typically above 6.0. Because dietary roughage increases rumen pH, a decrease in roughage would create a more acidic environment which is not optimal for fibrolytic bacteria (Dijkstra et al., 2005) but does not explain the reduction in ADF digestion. Luepp et al. (2009) observed similar results in ruminally and duodenally cannulated steers. An increase in DDGS resulted in a decrease in ruminal ADF digestion. Luepp et al. (2009) also observed that higher DDGS inclusion tended to have a quadratic effect on total tract ADF digestion, where steers fed 15% DDGS had the greatest total tract ADF digestion and steers fed 60% DDGS had the lowest total tract ADF digestion. In the current experiment, there was an effect of sulfur on ADF digestion. Because sulfur content is directly related to the concentration of DDGS in the diet, the effect of sulfur on ADF digestion could be due to the increase in indigestible fiber content of DDGS. There was no effect of dietary roughage, sulfur, or the interaction of dietary roughage and sulfur

on NDF digestion in the current experiment. This observation is in contrast with Luepp et al. (2009) who found a tendency for ruminal NDF digestion to decrease with higher inclusion of DDGS. However, results are consistent with data showing no effect of DDGS on total tract NDF digestion. A higher insoluble fiber content or difference in plant structure could account for the difference in ADF but not NDF digestion.

In experiment 1, there was no effect of roughage, sulfur or interaction between roughage and sulfur on apparent and true OM digestion. However, inclusion of DDGS resulted in a reduction in apparent OM digestion compared with the CON diet. A reduction in apparent OM digestion suggests a greater amount of effluent OM or less fermentable substrate available to rumen microbes (May et al., 2010). Luepp et al. (2009) observed similar results which showed an effect of DDGS inclusion resulting in a decrease in apparent and true OM digestion. This response was not observed in the current experiment; however, in the study by Luepp et al. (2009), they included diets containing up to 60% DDGS compared with only 40% in the current experiment. Similar results may have been observed if diets containing higher concentrations of DDGS were used in the current experiment. Corrigan et al. (2009) noted a reduction in total tract digestion of OM with the inclusion of 40% WDGS compared with a control diet. In the experiment, DDGS was added to achieve higher concentrations of sulfur, an inorganic mineral. Therefore, as DDGS concentration increased, the OM content decreased.

Increasing dietary sulfur had a tendency to decrease total VFA concentration. Because higher concentrations of dietary sulfur were achieved by increasing DDGS, this negative impact on total VFA concentration can be explained by a decrease in

fermentable substrate with DDGS compared with corn that it replaced (May et al., 2010; Leibovich et al., 2009). Dietary sulfur tended to have a negative effect on A:P ratio due to a decrease in the molar proportion of acetate. Sulfur-reducing bacteria oxidize acetate to CO_2, which could explain the decrease in the molar proportion of acetate (Appels et al., 2008). In addition, an increase in dietary sulfur resulted in an increase in the molar proportion of butyrate, in agreement with Luepp et al. (2009).

Inclusion of DDGS in the diet caused an increase in fermenter pH. Leupp et al. (2009) reported similar observations in ruminally and duodenally cannulated steers. A decrease in fermentable substrate with DDGS-containing dietary treatments could explain the increase in fermenter pH. Ammonia-N production was greater with higher inclusion of dietary sulfur but was not affected by dietary roughage or the interaction of dietary sulfur and roughage. Inclusion of DDGS resulted in lower NH_3-N production compared with CON, in contrast to results by Luepp et al. (2009). Rumen microbes can be very efficient at converting animal protein to bacterial protein, especially when provided distillers solubles (Hatch et al., 1972).

Nitrogen metabolism measurements in experiment 1 were affected by both dietary roughage and sulfur. There was a decrease in dietary-N flow and a tendency to increase microbial-N as dietary sulfur concentrations increased, suggesting an increase in dietary-N utilization by rumen microbes. These findings are consistent with Luepp et al. (2009) who observed similar results in total tract CP digestion. Efficiency of CP synthesis tended to be higher with DDGS-containing diets compared with the CON treatment. Luepp et al. (2009) found no effect of DDGS on EMPS using ruminally- and duodenally-

cannulated steers. Differences could be due to differences in experimental diet as well as innate differences between *in situ* and *in vitro* studies. Luepp et al. (2009) included urea and sunflower meal in their diet as protein sources and diets had varying CP concentrations (15 to 21.7%).

Experiment 2

In the second experiment, batch cultures were used to investigate whether dietary roughage could mitigate the production of H_2S production caused by DDGS inclusion in the diet without causing detrimental effects on microbial fermentation. In the current experiment, an increase in dietary sulfur elicited an increase in total H_2S production in batch culture fermentation, which is similar to observations by May et al. (2010) and Leibovich et al. (2009). However, this observation is in contrast to results found in experiment 1 where no effect of sulfur, roughage or the interaction between roughage and sulfur on H_2S production was found in batch culture. Production of H_2S numerically increased with addition of sulfur to the diet. Treatments were replicated (n = 3) per period in experiment 2 but were not (n = 1) replicated per period in experiment 1. The greater number of observations could account for possibly detecting significance in one experiment and not in the other. There was a tendency for total gas production to increase with added dietary sulfur, which is in disagreement with previous research by Smith et al. (2010) who observed no effect of sulfur on total gas production. Dietary sulfur concentrations used by Smith et al. (2010) were 0.2, 0.4 and 0.8% which is fairly similar to that used in the current experiment (0.3, 0.4 and 0.5%). While dietary sulfur concentrations are similar between studies, Smith et al. (2010) achieved higher sulfur

concentrations by addition of Na_2SO_4 solutions and in the current experiment, higher dietary sulfur was achieved by addition of DDGS in the diet. Because Smith et al. (2010) observed no effect of sulfur on total gas production, the effect could be due to sulfur source or differences in feedstuffs, among other variables, that differed between the two experiments. Smith et al. (2010) included urea and cottonseed meal in the diet.

Dietary roughage appeared to have a negative impact on microbial fermentation *in vitro*. An increase in final pH and change in pH were observed with additional dietary roughage inclusion, suggesting that higher roughage dietary treatments contained less fermentable substrate. In the current experiment, less fermentable substrate was achieved in two ways: 1) when DDGS was included in diets compared with CON diet containing 0% DDGS and 2) when greater inclusion of DDGS replaced corn. Dietary treatments utilized by Leibovich et al. (2009) also contained lower fermentable substrate with addition of distillers grains compared with control diets and also with an increase in DDGS between dietary treatments. Similar results for *in vitro* dry matter disappearance (IVDMD) have been observed by Leibovich et al. (2009). May et al. (2010) observed that dietary inclusion of 15% sorghum WDGS compared with 0% sorghum WDGS in a control diet resulted in a decrease in IVDMD. They also noted that an increase of WDGS from 15 to 30% of substrate DM decreased IVDMD. Therefore it can be reasoned that lower amounts of fermentable substrate result in lower total VFA production, which was observed in the current experiment.

In addition to lowering total VFA concentration, moderate dietary roughage treatments decreased the A:P ratio by depressing the molar proportion of acetate.

Because this was observed in experiment 2 and not in experiment 1, this could be due to either an increase in observations across periods providing greater power to detect significant differences or simply differences between short-term batch culture fermentation and more realistic continuous culture fermentation. Dietary sulfur also lowered the molar proportion of acetate. Because sulfur content is directly related to the DDGS concentration, these results are in agreement with previous research (Luepp et al., 2009; Vander Pol, 2009). However, those experiments also observed an increase in propionate with higher concentrations of DDGS, which was not observed in the current experiment. In addition, high dietary sulfur treatments had a higher molar proportion of butyrate compared with low and moderate dietary sulfur treatments, which was not observed by Luepp et al. (2009). Dietary roughage did not affect molar proportions of propionate or butyrate. It should also be noted that in the current experiment, there was lower total VFA production and A:P ratio with the inclusion of DDGS in the diet. As observed previously (May et al., 2010; Leibovich et al., 2009), this is likely due to the decrease in fermentable substrate in DDGS compared with corn-based diets.

There was no effect of roughage, sulfur, or the interaction of roughage and sulfur on NH_3-N production, which is in contrast with results from experiment 1 where an effect of dietary sulfur on NH_3-N production was observed.

CONCLUSIONS

While the current experiments did not find any beneficial effect of dietary roughage on mitigating ruminal H_2S production, results did confirm that an increase in dietary sulfur increased H_2S production. Inclusion of DDGS compared with corn-based

diets showed an overall decrease in total VFA concentration and molar proportions of acetate, thereby resulting in a decrease in the A:P ratio. However, this is most likely due to a decrease in fermentable substrate in DDGS compared with corn-based diets and not because of the increase in dietary sulfur content. However, in some aspects, DDGS inclusion was beneficial as substrate for ruminal microbes with greater ADF and true OM digestion. Nitrogen data revealed that DDGS is a quality feedstuff that can be utilized by rumen microbes as an effective CP source. Although dietary roughage did not have the desired effect in the current experiment, other forms of dietary manipulation may be effective in mitigating H_2S production. Further research needs to be conducted to evaluate other options (i.e. sources of roughage, concentrations of roughage, additives, binders, etc.) in controlling ruminal H_2S production.

Table 1. Ingredient and chemical composition of experimental diets[1]

Item	CON	LRLS	LRMS	LRHS	MRLS	MRMS	MRHS
Feed composition[2]							
Corn, ground	74.9	52.1	52.5	52.9	47.7	48.1	48.5
High sulfur DDGS[3]	0.0	7.0	23.0	40.0	7.0	23.0	40.0
Low sulfur DDGS[3]	0.0	33.0	17.0	0.0	33.0	17.0	0.0
Grass hay	9.0	3.0	3.0	3.0	9.0	9.0	9.0
R500 supplement[4]	7.0	0.0	0.0	0.0	0.0	0.0	0.0
R1500 supplement[5]	0.0	2.3	2.3	2.3	2.3	2.3	2.3
Soybean meal	8.8	2.3	1.9	1.5	0.8	0.4	0.0
Calcium sulfate	0.20	0.20	0.20	0.20	0.20	0.20	0.20
Calcium carbonate	0.13	0.08	0.07	0.07	0.00	0.00	0.00
Chemical composition[2]							
DM	91.7	93.0	92.5	92.5	93.0	92.9	92.9
CP	17.4	17.9	18.1	17.9	17.6	17.5	17.5
ADF	8.2	8.5	8.3	8.5	9.4	9.0	10.5
NDF	12.9	18.6	19.4	17.9	23.8	20.8	19.9
Sulfur	0.22	0.27	0.36	0.44	0.27	0.35	0.46

[1] CON = Control, no DDGS, 9% roughage, 0.18% S; LRLS = 3% roughage, 0.30% S; LRMS = 3% roughage, 0.40% S; LRHS = 3% roughage, 0.50% S; MRLS = 9% roughage, 0.30% S; MRMS = 9% roughage, 0.40% S; MRHS = 9% roughage, 0.50% S. All treatments other than CON contained 40% DDGS.

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

[3] DDGS=Dried distillers grains with solubles.

[4] Steakmaker R500 mineral mix contained 99.1% DM, 65.2% CP, 13.3% ADF, 20.8% NDF and 0.11% S.

[5] Steakmaker R1500 mineral mix contained 99.1% DM, 1.4% CP, 13.3% ADF, 20.8% NDF and 0.11% S.

Table 2. Effects of dietary roughage and sulfur concentrations on hydrogen sulfide production and digestion in continuous cultures of rumen contents

	DDGS dietary treatments[1]											
	Roughage		Sulfur[7]			SEM[2]	*P*-value[3]			Contrast effect[4]		*P*-value[3]
Item	LR	MR	LS	MS	HS		R	S	R x S	CON	DDGS[5]	S
H_2S production (µg)[6]	1.19	2.03	0.95	1.82	2.05	0.94	0.14	0.25	0.75	0.13	1.61	0.04
H_2S (µg/mL)	0.40	0.68	0.32	0.61	0.68	0.31	0.14	0.25	0.75	0.04	0.54	0.04
Digestion (g/100 g)												
OM, apparent	32.4	25.3	27.2	26.8	32.4	1.6	0.30	0.25	0.48	32.4	26.4	< 0.01
OM, true	53.7	50.3	45.6	58.3	52.1	7.8	0.47	0.10	0.90	55.1	52.0	0.59
NDF	7.7	9.8	10.1	8.3	7.8	3.8	0.48	0.79	0.29	5.6	17.5	0.38
ADF	35.2	23.1	26.5[a]	24.8[a]	36.1[b]	5.8	< 0.01	0.03	0.70	31.2	29.0	0.74

[1]LR = low roughage, 3% roughage; MR = moderate roughage, 9% roughage; LS = low sulfur, 0.3% sulfur; MS = moderate sulfur, 0.4% sulfur; HS = high sulfur, 0.5% sulfur. All treatments other than CON contained 40% DDGS.

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

[3] Probability corresponding to the null hypothesis where R is dietary roughage, S tests the linear comparison of dietary sulfur and R x S is the interaction effect.

[4] Statistical contrast of control diet against DDGS-containing diets where DDGS is average value of DDGS-containing diets.

[5] Average of six DDGS-containing dietary treatments. Treatment results are reported as least squares means.

[6] Hydrogen sulfide production from 3 mL subsample.

[7] Differing superscripts indicate differences between treatments.

Table 3. Effects of dietary roughage and sulfur concentrations on pH in continuous cultures of rumen contents

	DDGS dietary treatments[1]						SEM[2]	*P*-value[3]			Contrast effect[4]		*P*-value[3]
Item	LRLS	LRMS	LRHS	MRLS	MRMS	MRHS		R	S	R x S	CON[1]	DDGS[5]	S
pH	5.12[a]	5.21[b]	5.17[c]	5.16[c]	5.21[b]	5.23[b]	0.01	< 0.01	< 0.01	< 0.01	5.09	5.18	< 0.01

[1] CON = Control, no DDGS, 9% roughage, 0.18% S; LRLS = 3% roughage, 0.30% S; LRMS = 3% roughage, 0.40% S; LRHS = 3% roughage, 0.50% S; MRLS = 9% roughage, 0.30% S; MRMS = 9% roughage, 0.40% S; MRHS = 9% roughage, 0.50% S. All treatments other than CON contained 40% DDGS.

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

[3] Probability corresponding to the null hypothesis where R is dietary roughage, S tests the linear comparison of dietary sulfur and R x S is the interaction effect.

[4] Statistical contrast of control diet against DDGS-containing diets where DDGS is average value of DDGS-containing diets.

[5] Average of six DDGS-containing dietary treatments. Treatment results are reported as least squares means.

Table 4. Effects of dietary roughage and sulfur concentrations on volatile fatty acid parameters in continuous cultures of rumen contents

	DDGS dietary treatments[1]											
	Roughage		Sulfur[6]			SEM[2]	*P*-value[3]			Contrast effect[4]		*P*-value[3]
Item	LR	MR	LS	MS	HS		R	S	R x S	CON	DDGS[5]	S
Total VFA, m*M*	154.2	146.4	158.0	157.3	135.6	15.6	0.40	0.10	0.38	203.7	149.1	< 0.01
Total branched-chain VFA, m*M*	0.9	0.8	0.9	0.8	0.9	0.2	0.15	0.89	0.25	1.3	0.9	0.04
A:P ratio	3.2	3.0	3.2	3.2	2.8	0.2	0.19	0.09	0.76	4.1	3.0	< 0.01
Individual VFA, mol/100 mol												
Acetate	60.5	58.4	62.5[a]	60.9[a]	54.8[b]	2.3	0.14	< 0.01	0.94	68.2	58.7	< 0.01
Propionate	19.3	19.9	19.5	19.5	19.8	0.9	0.32	0.82	0.35	16.7	20.1	< 0.01
Butyrate	16.2	17.1	14.5[a]	15.4[a]	20.0[b]	1.5	0.32	< 0.01	0.40	12.5	16.7	< 0.01
Isobutyrate	0.2	0.2	0.2	0.2	0.2	0.1	0.82	0.91	0.72	0.2	0.2	0.70
Valerate	1.4	1.6	1.3[a]	1.3[a]	1.8[b]	0.2	0.13	< 0.01	0.88	1.2	1.5	0.13
Isovalerate	0.4	0.3	0.4	0.3	0.4	0.1	0.10	0.08	0.20	0.4	0.4	0.63
Caproate	2.0	2.6	1.7[ab]	2.4[a]	2.9[b]	0.3	0.01	< 0.01	0.33	0.8	2.5	< 0.01
Branched-chain VFA	0.6	0.5	0.5[a]	0.5[b]	0.6[b]	0.1	0.30	0.31	0.45	0.6	0.6	0.61

[1]LR = low roughage, 3% roughage; MR = moderate roughage, 9% roughage; LS = low sulfur, 0.3% sulfur; MS = moderate sulfur, 0.4% sulfur; HS = high sulfur, 0.5% sulfur. All treatments other than CON contained 40% DDGS.

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

[3] Probability corresponding to the null hypothesis where R is dietary roughage, S tests the linear comparison of dietary sulfur and R x S is the interaction effect.

[4] Statistical contrast of control diet against DDGS-containing diets where DDGS is average value of DDGS-containing diets.

[5] Average of six DDGS-containing dietary treatments. Treatment results are reported as least squares means.

[6] Differing superscripts indicate differences between treatments.

Table 5. Effects of dietary roughage and sulfur concentrations on nitrogen metabolism in continuous cultures of rumen contents

	DDGS dietary treatments[1]									Contrast effect[4]		*P*-value[3]
	Roughage		Sulfur[7]			SEM[2]	*P*-value[3]					
Item	LR	MR	LS	MS	HS		R	S	R x S	CON	DDGS[5]	S
NH_3-N (mg/dL)	1.69	1.84	1.15[a]	1.37[a]	2.76[b]	0.43	0.56	< 0.01	0.34	4.75	1.76	< 0.01
Nitrogen flow (g/d)												
NH_3-N	0.03	0.04	0.02[a]	0.03[a]	0.06[b]	0.01	0.64	< 0.01	0.51	0.09	0.03	< 0.01
Non NH_3-N	2.40	2.31	2.40	2.33	2.33	0.06	0.02	0.26	0.53	2.22	2.35	< 0.01
Microbial-N	1.62	1.45	1.22[a]	1.67[b]	1.73[b]	0.30	0.33	0.05	0.85	1.40	1.54	0.53
Dietary-N	0.79	0.86	1.19[a]	0.67[b]	0.62[b]	0.30	0.68	0.03	0.78	0.82	0.83	0.11
CP degradation, %	63.4	59.1	44.3[a]	68.6[b]	70.8[b]	14.0	0.60	0.03	0.78	60.7	62.2	0.12
EMPS[6]	42.2	40.3	37.7[a]	39.3[a]	46.7[b]	5.4	0.41	0.01	0.81	36.1	41.3	0.09

[1]LR = low roughage, 3% roughage; MR = moderate roughage, 9% roughage; LS = low sulfur, 0.3% sulfur; MS = moderate sulfur, 0.4% sulfur; HS = high sulfur, 0.5% sulfur. All treatments other than CON contained 40% DDGS.

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

[3] Probability corresponding to the null hypothesis where R is dietary roughage, S tests the linear comparison of dietary sulfur and R x S is the interaction effect.

[4] Statistical contrast of control diet against DDGS-containing diets where DDGS is average value of DDGS-containing diets.

[5] Average of six DDGS-containing dietary treatments. Treatment results are reported as least squares means.

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

[7] Differing superscripts indicate differences between treatments.

Table 6. Effects of dietary roughage and sulfur concentrations on ammonia nitrogen concentration, hydrogen sulfide, gas production and pH in batch cultures of rumen contents

	DDGS dietary treatments[1]					SEM[2]						
	Roughage		Sulfur[6]				*P*-value[3]			Contrast effect[4]		*P*-value[3]
Item	LR	MR	LS	MS	HS		R	S	R x S	CON	DDGS[5]	S
NH_3-N (mg/dL)	4.30	4.33	3.85	4.17	4.92	0.65	0.96	0.18	0.57	4.21	4.32	0.84
Total H_2S production (µg)	58.0	71.7	42.2[a]	70.5[ab]	81.9[b]	20.0	0.25	0.04	0.60	21.2	64.9	< 0.01
H_2S (µg/mL)	1.60	1.94	1.18[a]	1.94[ab]	2.19[b]	0.52	0.27	0.04	0.55	0.56	1.77	< 0.01
Total gas (mL)	36.2	36.6	35.8	36.0	37.4	1.0	0.50	0.09	0.14	38.9	36.4	0.02
Initial pH	5.24	5.28	5.19	5.27	5.33	0.10	0.54	0.14	0.10	5.09	5.26	0.03
Final pH	5.61	5.71	5.61	5.66	5.71	0.06	0.01	0.08	0.11	5.44	5.66	< 0.01
Change in pH	0.36	0.43	0.43	0.39	0.38	0.07	0.08	0.60	0.25	0.35	0.40	0.37

[1]LR = low roughage, 3% roughage; MR = moderate roughage, 9% roughage; LS = low sulfur, 0.3% sulfur; MS = moderate sulfur, 0.4% sulfur; HS = high sulfur, 0.5% sulfur. All treatments other than CON contained 40% DDGS.

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

[3] Probability corresponding to the null hypothesis where R is dietary roughage, S tests the linear comparison of dietary sulfur and R x S is the interaction effect.

[4] Statistical contrast of control diet against DDGS-containing diets where DDGS is average value of DDGS-containing diets.

[5] Average of six DDGS-containing dietary treatments. Treatment results are reported as least squares means.

[6] Differing superscripts indicate differences between treatments.

Table 7. Effects of dietary roughage and sulfur concentrations on volatile fatty acid parameters in batch cultures of rumen contents

	DDGS dietary treatments											
	Roughage		Sulfur[6]			SEM[2]	*P*-value[3]			Contrast effect[4]		*P*-value[3]
Item	**LR**	**MR**	**LS**	**MS**	**HS**		**R**	**S**	**R x S**	**CON**	**DDGS[5]**	**S**
Total VFA, m*M*	134.7	119.1	124.7	132.0	124.0	5.2	< 0.01	0.33	0.22	153.3	125.6	< 0.01
Total branched-chain VFA,	2.2	1.8	1.7	1.9	2.4	0.5	0.22	0.17	0.79	1.7	2.1	0.45
A:P ratio	2.6	2.3	2.7[a]	2.6[a]	2.1[b]	0.3	0.10	0.02	0.78	3.2	2.4	< 0.01
Individual VFA, mol/100 mol												
Acetate	55.8	51.5	58.3[a]	55.9[a]	46.6[b]	4.2	0.10	< 0.01	0.92	61.8	53.1	< 0.01
Propionate	22.0	22.5	21.8	22.0	22.9	1.1	0.41	0.35	0.44	19.6	22.6	0.03
Butyrate	15.7	18.2	14.3[a]	15.7[a]	20.9[b]	2.6	0.11	< 0.01	0.77	14.0	16.8	0.22
Isobutyrate	0.5	0.5	0.4[a]	0.5[a]	0.6[b]	0.1	0.20	< 0.01	0.44	0.3	0.5	< 0.01
Valerate	1.6	2.0	1.5[a]	1.6[a]	2.2[b]	0.3	0.08	0.02	0.65	1.8	1.7	0.96
Isovalerate	1.1	1.0	0.9	1.0	1.3	0.3	0.62	0.13	0.81	0.8	1.2	0.17
Caproate	3.4	4.3	2.7[a]	3.4[a]	5.4[b]	0.8	0.07	< 0.01	0.99	1.7	4.1	< 0.01
Branched-chain VFA	1.8	1.5	1.3[a]	1.5[ab]	1.9[b]	0.3	0.83	0.04	0.75	1.1	1.7	0.06

[1]LR = low roughage, 3% roughage; MR = moderate roughage, 9% roughage; LS = low sulfur, 0.3% sulfur; MS = moderate sulfur, 0.4% sulfur; HS = high sulfur, 0.5% sulfur. All treatments other than CON contained 40% DDGS.

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

[3] Probability corresponding to the null hypothesis where R is dietary roughage, S tests the linear comparison of dietary sulfur and R x S is the interaction effect.

[4] Statistical contrast of control diet against DDGS-containing diets where DDGS is average value of DDGS-containing diets.

[5] Average of six DDGS-containing dietary treatments. Treatment results are reported as least squares means.

[6] Differing superscripts indicate differences between treatments.

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