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

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ABSTRACT: In ruminants, excess dietary sulfur can be associated with a reduction in DM intake, poor feedlot performance and sulfur-associated polioencephalomalacia. Bismuth subsalicylate (BSS) has been shown to decrease hydrogen sulfide in vitro. The objective of this experiment was to evaluate effects of BSS inclusion (0 or 0.5% of diet DM) and dietary sulfur (0.21 or 0.42% of diet DM) on microbial fermentation in continuous culture. Treatments were arranged in a 2 × 2 factorial design. Eight dual-flow continuous culture fermenters were used during 2 consecutive 10-d periods consisting of 7 d for stabilization followed by 3 d of sampling. A pelleted feedlot diet containing 39% dry rolled corn, 32% earlage, 21% wet distillers grains, 3.2% corn silage, 1.5% soybean meal, 0.6% urea and 2.7% mineral premix (DM basis) was provided as substrate for microbes at a rate of 75 g of DM × fermenter^{-1} × d^{-1}. Effluents from sampling days were composited by fermenter within period, resulting in 4 replicates/treatment. Bismuth subsalicylate inclusion decreased ($P < 0.01$) true OM digestion, while no effects were observed for NDF and ADF digestion. Total VFA concentrations, molar proportions of acetic, propionic, and branched-chained VFA decreased ($P < 0.01$) with BSS addition. The ratio of acetic to propionic acid and the molar proportion of butyric acid increased ($P < 0.01$) with BSS addition. In regard to nitrogen metabolism, BSS increased NH_3–N concentration, NH_3–N and dietary-N flows ($P < 0.01$), and decreased non-NH_3–N flow, microbial-N flow, CP degradation, and efficiency of microbial protein synthesis ($P < 0.01$). Inclusion of BSS increased mean, minimum, and maximum fermentation pH ($P < 0.01$). Amount of dietary sulfur and BSS inclusion influenced flows of amino acids and fatty acids from fermenters. Influences on fatty acid biohydrogenation and amino acid flows demonstrated an overall suppression of microbial fermentation. Results from this experiment indicate that BSS inclusion at 0.5% of diet DM has detrimental effects on in vitro rumen fermentation in continuous culture.

Key words: bismuth subsalicylate, continuous culture, fermentation, rumen

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INTRODUCTION

Intake of excessive dietary sulfur in ruminant animals is associated with several undesirable conditions including a reduction in DM intake, decreases in growth performance, poor carcass characteristics (Lonergan et al., 2001) and an increase in the incidence of sulfur associated polioencephalomalacia (PEM; Gould, 1998). Dietary sulfur is reduced to hydrogen sulfide by bacteria such as *Desulfobrivio* and *Desulphotomaculum* (Russell, 2002). Vanness et al. (2009) concluded that low rumen pH conditions asso-

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ciated with low dietary roughage favored the reduction of sulfate to H_2S. This situation is a concern in feedlot cattle fed distiller's grains, where ruminal pH is low and dietary sulfur is high. Hydrogen sulfide is normally absorbed through the rumen wall and detoxified in the liver; however, eructation of gaseous H_2S can lead to re-inhalation by the animal, thereby bypassing hepatic detoxification and increasing the risk for PEM (Gould, 1998). Bismuth subsalicylate (BSS) is a compound commonly used in humans to decrease hydrogen sulfide accumulation in the lower gut (Suarez et al., 1998). Ruiz-Moreno et al. (2015) demonstrated strong reductions in H_2S accumulation in batch culture when BSS was included at 0.5, 1, 2, and 4% of diet DM. Inclusion of BSS at 1% of diet DM in a continuous culture system markedly reduced H_2S production; however total VFA concentration, CP degradation and microbial protein synthesis also decreased with BSS addition.

Previous research in continuous culture only investigated BSS inclusion at 1% of diet DM, and more research is needed to understand the effects of a lower inclusion of BSS. Data from the dose titration experiment of Ruiz-Moreno et al. (2015) indicated that 0.5% may not be as detrimental to overall fermentation. Effects of BSS on amino acid and fatty acid metabolism have not been investigated in previous work on the compound. Furthermore, research is needed on possible interactions of BSS and S level, including possible reduction in microbial fermentation due to irreversible binding of S.

Therefore, the objective of this study is to determine effects of BSS inclusion at 0.5% of diet DM with 2 levels of dietary sulfur (0.21 or 0.42% of diet DM) on in vitro fermentation with rumen microbes. Primary emphasis was on BSS induced alterations of fermentation, including fatty and amino acid metabolism and secondary emphasis was on the influence of the interaction of BSS with dietary sulfur level on microbial fermentation.

MATERIALS AND METHODS

Animals and Collection of Rumen Fluid

The University of Minnesota Institutional Animal Care and Use Committee approved all animal use in this study (ACUP # 0706A09602). All animals involved in the study were cared for according to the specific guidelines and recommendations outlined by the Care and Use of Animals in Agricultural Research (FASS, 2010). Two ruminally cannulated lactating dairy cows served as rumen fluid donors. The diet fed to donor cows was formulated to meet or exceed requirements of a Holstein cow producing 40 kg of milk/d with 3.7% fat and 3.3% protein (NRC, 2001). Rumen contents from each cow were transferred to a pre-warmed container for transport to the laboratory. Contents from both cows were combined in equal parts and strained through 4 layers of cheesecloth. Strained fluid was equally divided into 8 pre-warmed fermenters (1,040 ± 20 mL strained fluid per fermenter). Twenty-five grams of pelleted experimental diet were added to fermenters immediately after inoculation.

Experimental Diets

A basal diet was formulated to meet or exceed requirements for finishing feedlot cattle (NRC, 2000). Ingredient and chemical composition of the diet is shown in Table 1, and fatty acid and amino acid composition are show in Table 2. All diet ingredients were mixed in a stationary mixer. The basal diet was dried at 60°C in a forced air oven for 48 h and ground in a Wiley No. 4 laboratory mill (Arthur H. Thomas Co., Philadelphia, PA) to pass a 2-mm screen. The ground diet was split into 2 equal portions and sodium sulfate was added to 1 portion to achieve a dietary sulfur content of 0.42%. Sulfur content of the basal diet was 0.21%. The high and low sulfur portions were then each divided a second time, and bismuth subsalicylate (Sigma-Aldrich No. 480789, St. Louis, MO) was added to 1 portion to achieve concentration of 0.5% of diet DM as BSS. The portion of the high and low sulfur diets without BSS addition served as a control (CON)

Table 1. Ingredient and chemical composition of basal experimental diet

Item	Composition[1]
Ingredient composition	
Dry rolled corn	39.0
Earlage	32.1
Wet distiller's grains	21.2
Corn silage	3.2
Supplement[2]	2.6
Urea premix[3]	2.0
Chemical composition	
Crude protein	15.9
NDF	20.6
ADF	8.6
Lignin	1.0
Ash	5.3
Starch	45.8
Crude fat	5.1
Diet NEg, Mcal/kg[4]	1.4

[1]Composition as % of 100 °C DM.

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

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

[4]Estimated using Nutrient Requirements for Beef Cattle (NRC, 2000).

Table 2. Fatty acid and amino acid composition of the basal diet

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

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

within each sulfur level. Diet preparation resulted in 4 treatments arranged in a 2 × 2 factorial design.

Each treatment diet was pelleted with a CL-5 California Pellet mill (California Pellet Mill Co., Crawfordsville, IN) to a final pellet dimension of 6 mm diameter × 12 mm long. Pelleting was performed to facilitate use of an automated feed delivery system to the fermenters. Pelleted diets were placed in shallow trays and allowed to air dry for 96 h before storing in plastic containers.

Continuous Culture Operation

Eight dual flow continuous culture fermenters, described by Hannah et al. (1986) with a modified pH control and measuring system were used in 2 consecutive 10-d experimental periods. Treatments were randomly assigned and duplicated within block (experimental period) to create a randomized complete block design with 4 observations per treatment. Fermenters were provided with pelleted diet divided into 8 equally spaced 90-min feedings using an automated feeding system. Amount of diet (as-fed) was adjusted on d 0, 4, and 7 for DM content to attain a feeding rate of 75 g of diet DM/d.

Artificial saliva (pH = 8.25) was prepared according to Weller and Pilgrim (1974) except for the replacement of $MgSO_4$ with $MgCl_2$ to a final composition (g/L) 5.0 g $NaHCO_3$, 1.76 g Na_2HPO_4, 1.6 g $KHCO_3$, 0.6 g KCl, 0.05 g $MgCl_2$, and 0.4 g urea. Liquid flow rate of fermenters was set at 8.2% of fermenter volume (1,040 ± 20 mL) per h by regulating artificial saliva input. Solids dilution rate was set at 4.1%/h by regulating liquid output through filters. Individual fermenter pH was measured every 5 min by an electric acquisition system (DasyLab v.5.04, DASYTEC USA, Bedford, NH). Fermenter pH was maintained between 5.1 and 5.8 by automated addition of either 5*M* NaOH or 3*M* HCl. Anaerobic conditions were maintained with constant infusion of N_2 at a rate of 20 mL/min using a digital flow meter (Aalborg GFM 17, Orangeburgh, NY).

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

Sample Collection

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

Chemical Analysis

Diets, lyophilized effluent and lyophilized microbial cells were analyzed for DM and ash by drying in an oven at 105°C for 24 h followed by combustion at 550°C for

6 h (AOAC, 2005; method 934.01). Sequential detergent fiber analysis was conducted to determine NDF and ADF concentrations of diets and effluents using an ANKOM A200 fiber analyzer with F57 fiber bags (ANKOM Corp, Fairport, NY) and lignin content of the diet was measured gravimetrically after hydrolysis of acid detergent residue using 12 *M* H_2SO_4 (Van Soest, 2015). Nitrogen content of the diets, liquid and freeze dried effluent, microbial cells and acid detergent residue were determined using the Kjeldahl method (AOAC, 1990; method 984.13). Purine concentration of effluent and microbial pellet was used to partition flows of effluent N into microbial and dietary N (Zinn and Owens, 1986). Ammonia-N concentration in the fermenters was determined on the supernatant of a centrifuged (5,000 × *g* for 15 min at 4°C) subsample of liquid effluent by steam distillation (Bremner and Keeney, 1965) with magnesium oxide using a 2,300 Kjeltec Analyzer Unit (Foss Tecator AB, Höganäs, Sweden).

Effluent VFA concentration was determined using gas-liquid chromatography. Fermenter effluent was clarified by centrifugation at 5,000 × *g* at 4°C for 10 min. Two milliliters of supernatant was hydrolyzed using 0.5 mL of 25% (wt/vol) m-phosphoric acid. The sample was frozen at –20°C and thawed, followed by additional centrifugation at 5,000 × *g* at 4°C for 10 min to remove hydrolyzed compounds. Clarified fluid was filtered through a polyethersulfone micropore filter with a 0.45 μm pore size. Analysis was performed using an HP 6890 GC (Hewlett-Packard, Palo Alto, CA) equipped an autosampler and a Supelco Carbopack glass column (2 m × 6.35 mm × 2 mm). Chromatographic conditions were as follows: injection volume: 1.0 μL; injector temperature: 200°C; nitrogen flow (carrier gas): 24.6 mL/min; oven settings: initial temperature at 175°C, held for 21 min, ramped at 25°C/min to 200°C, held for 4 min, post run cool down held at 175°C for 4 min; flame ionization detector temperature: 230°C. Standard solutions with known concentrations of VFA were analyzed to calibrate chromatograph output and determine concentration of VFA in samples.

Amino acid content (excluding tryptophan) of lyophilized effluent was determined by the University of Missouri Agricultural Experiment Station according to official method 994.12 (AOAC, 2006). Fatty acid analysis was performed by T. C. Jenkins at the University of Clemson (Jenkins, 2010). Sodium methoxide and methanolic HCl were used for direct *trans*-esterification of fatty acids to methyl esters in lyophilized effluent samples. Fatty acid methyl esters were separated using an HP 5890A GLC (Hewlett-Packard, Palo Alto, CA) equipped with an autosampler and an SP. 2380 fused-silica capillary column (100 m × 0.25 mm i.d., 0.2-μm film thickness). Chromatographic conditions were: helium flow (carrier and make-up gas): 20 mL/min; oven settings: initial temperature at 140°C, held for 4 min, ramped at 13°C/min to 160°C, held for 44 min, ramped at 4°C/min to 220°C, held for 20 min; flame ionization detector temperature: 250°C. Methyl ester peaks were identified based on comparison of their retention times to those of pure standards.

Statistical Analysis

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

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

Where μ is the grand mean, P_i is the period (block), B_j is the BSS level, S_k is the sulfur level, $B_j \times S_k$ is the interaction term and ε_{ijk} is the random error. For all analysis, differences were considered significant at $P \leq 0.05$, with tendencies discussed at $0.01 < P \leq 0.1$. Differences among treatments were tested using LSMEANS with the PDIFF option in SAS. Results are reported as least squared means from 4 observations per treatment unless otherwise noted.

Fermentation pH recorded every 5 min obtained over 3 sampling days were summarized to determine simple mean, minimum and maximum pH on an hourly basis. These hourly statistics were analyzed as repeated measures using the MIXED procedure of SAS software with a compound symmetry covariance structure. This covariance structure had the lowest AIC (Akaike's Information Criterion) value of several competing structures (Littell et al., 1998). Fixed effects in the model were dietary sulfur, BSS, and the interaction between the 2 factors. Random effect was fermenter nested within period.

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

RESULTS AND DISCUSSION

Dry Matter, Organic Matter, and Fiber Digestion

Inclusion of BSS in the diet decreased ($P < 0.01$) true DM and OM digestion in continuous culture com-

pared with CON diets (Table 3). Neutral and acid detergent fiber digestion were not affected by treatment ($P > 0.05$). These results differ from Ruiz-Moreno et al. (2015) who observed increased true OM digestion and fiber digestion with BSS in the diet. Values obtained by Ruiz-Moreno et al. (2015) for NDF digestion were lower than ADF digestion and results from the present study display a similar inversion of NDF and ADF digestibilities. The same detergent fiber methodology was employed in both studies. These unrealistic relationships may be due to loss of fine particles after AD extraction of the effluent samples. Raffrenato and Van Amburgh (2011) demonstrated an increase in AD residue after addition of glass microfiber filters (1.5 µm pore size) to coarse Gooch crucibles (40 to 60 µm pore size). Alteration of the original Goering and Van Soest (1970) procedure increased AD residue measured in fecal samples by approximately 2% (Raffrenato and Van Amburgh, 2011). Greater loss of fines from effluent samples *vs.* the original feed may partially explain abnormalities in NDF *vs.* ADF digestion values in the present study. Overall, fiber digestion is of relatively lower concern in feedlot diets. However, the marked decreases in OM digestion in the present study indicate that BSS has the potential to negatively influence feed utilization.

While methodological considerations may be responsible for unrealistic NDF *vs.* ADF digestibilities, low overall fiber digestion values in this study are likely due to a decrease in fermentation pH. Hoover (1986) demonstrated that a reduction in pH had negative effects on fiber digestion while Yang et al. (2002) reported dramatic increases in fiber digestion as fermentation pH was artificially raised from 5.5 to 6.0. The low fermentation pH of CON diets (mean pH: 5.24) in the present study likely reduced fibrolytic activity in the fermenters. It is interesting to note higher fermentation pH associated with BSS inclusion did not result in concomitant enhancement of fiber digestion probably due to suppression of overall microbial activity associated with BSS inclusion.

VFA Concentration

Fermenters fed diets containing BSS displayed a large decrease ($P < 0.01$) in total VFA concentration and branched-chain VFA compared with fermenters fed CON diets (Table 4). Ruiz-Moreno et al. (2015) recorded a more modest decrease with BSS inclusion on total VFA concentration. In the present study, molar proportions of acetate and propionate decreased ($P < 0.01$) with BSS addition, while the molar proportion of butyric acid increased ($P < 0.01$), indicating a possible selective repression of acetate and propionate producers relative to butyrate producers. These findings are in contrast with Ruiz-Moreno et al. (2015), who observed increases in acetate and propionate and a decrease in butyrate molar proportions. These contradictory results between studies make it difficult to establish a possible mode of action of BSS. Discrepancies between Ruiz-Moreno et al. (2015) and the current study are possibly due to different levels of BSS inclusion and its effects on OM and fiber digestion between experiments. Ruiz-Moreno et al. (2015) had a greater concentration of distiller's grains, and a much different source of fiber than the present study. Dietary sulfur level and interaction between sulfur and BSS in the current study had no influence on any VFA measurements. This is in contrast to Quinn et al. (2009) who demonstrated that dietary sulfur level can alter the response of VFA proportions to monensin in continuous culture; however, results from Ruiz-Moreno et al. (2015) indicated that BSS and monensin had very different effects on rumen microbes in continuous culture.

Fermentation pH

Mean, minimum, and maximum fermentation pH increased ($P < 0.01$) with addition of BSS to the diet

Table 3. Effect of bismuth subsalicylate and dietary sulfur level on DM, OM and fiber digestion in continuous culture

	Treatment[1]							
	Low sulfur		High sulfur			*P*-value		
Item, g/100 g	CON	BSS	CON	BSS	SEM[2]	BSS	Sulfur	B × S
DM, apparent	47.1	39.2	50.3	39.3	1.4	< 0.01	0.31	0.38
DM, true[3]	64.1	46.9	67.9	47.6	2.5	< 0.01	0.13	0.33
OM, apparent	37.5	26.9	41.4	28.2	1.8	< 0.01	0.13	0.46
OM, true[3]	52.8	34.1	57.2	35.9	2.7	< 0.01	0.13	0.33
NDF	19.3	27.5	24.4	30.0	4.5	0.15	0.41	0.77
ADF	32.1	35.0	36.7	38.2	2.9	0.47	0.21	0.81

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

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

[3]Corrected for microbial contribution.

Table 4. Effect of bismuth subsalicylate and dietary sulfur level on VFA concentration and nitrogen metabolism in continuous culture

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

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

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

(Table 5). Mean fermentation pH was 5.73 for BSS diets, and 5.24 for CON diets. Dietary sulfur content had no effect on mean, minimum, or maximum fermentation pH ($P > 0.05$). Although fermentation pH was monitored and maintained between 5.1 and 5.8 by addition of 3 *M* HCl or 5 *M* NaOH, values that approached upper or lower limits were not observed and amount of acid or base pumped into fermenters during sampling days was very low (< 3 mL/d). The pH of initial inoculum from the rumen of donor cows at the start of period 1 and 2 was 5.3 and 5.2, respectively. Inclusion of BSS in the study by Ruiz-Moreno et al. (2015) resulted in similar effects on fermentation pH. Fermenter pH values in this experiment were slightly lower, which may relate to the higher concentration of VFA observed in the present study.

Table 5 also displays the percent of time fermenters spent within specified ranges of pH < 5.2, 5.2 < pH > 5.6, and pH > 5.6. Inclusion of BSS greatly increased ($P < 0.01$) time spent above pH 5.6 while fermenters without BSS (CON) spent a greater ($P = 0.03$) percent of time below pH 5.2. No fermenters with BSS recorded a pH below 5.2 throughout the duration of the experiment. Fermentation pH of fermenters fed CON diets spent a greater ($P < 0.01$) percent of time in the pH range of 5.2 to 5.6. Dietary sulfur had no effect ($P > 0.05$) on time spent within specified ranges of fermentation pH. Specific pH ranges were chosen to reflect physiologically relevant ranges, due to increasing risk of sub-acute ruminal acidosis as pH decreases below 5.6, with clinical signs of acute acidosis more common below pH 5.2 (Garrett et al., 1999). Fermentation pH could be considered low in the present study; however, it is important to note that inconsistent feeding behavior increases risk of acidosis (Owens et al., 1998; Schwartzkopf-Genswein et al., 2003; Nagaraja and Lechtenberg, 2007) and feeding in the current study was consistent due to the use of an automated system.

Table 5. Effect of bismuth subsalicylate (BSS) and dietary sulfur level on fermentation pH in continuous culture

	Treatment[1]							
	Low sulfur		High sulfur			*P*-value		
Item	CON	BSS	CON	BSS	SEM[2]	BSS	Sulfur	B × S
Mean pH[3]	5.23	5.75	5.25	5.72	0.02	< 0.01	0.77	0.26
Minimum pH[3]	5.17	5.70	5.19	5.68	0.03	< 0.01	0.95	0.50
Maximum pH[3]	5.30	5.80	5.32	5.77	0.02	< 0.01	0.81	0.20
Time above pH 5.6[4]	0.1	99.1	0.0	88.4	11.2	< 0.01	0.31	0.37
Time between pH 5.2 and 5.6[4]	74.3	0.9	76.2	11.6	21.2	< 0.01	0.58	0.68
Time below pH 5.2[4]	25.6	0.0	23.8	0.0	17.9	0.03	0.93	0.93

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

[2]Standard error of the mean.

[3]Analyzed as repeated measures with 1 observation^{-1} × fermenter^{-1} × hour during 3 consecutive sampling days.

[4]Expressed as percent of total time over 3 consecutive sampling days, n = 4 replicates per treatment.

Nitrogen Metabolism

Addition of BSS at 0.5% of the diet had a marked impact on fermenter nitrogen metabolism, while dietary sulfur level had no effects ($P > 0.05$; Table 6). Ammonia-N concentration in fermenter effluent increased ($P < 0.01$) from approximately 2.5 to 20.5 mg/dL with BSS inclusion in the diet. Ammonia-N concentrations of CON diets were very close to the minimum concentration of 2 mg NH_3–N/dL to support efficient microbial growth as recommended by Satter and Slyter (1974). Despite low NH_3–N concentrations, OM digestion and EMPS values were within the range of previous studies with similar levels of CP and CP degradability (Bach and Stern, 1999; Ariza et al., 2001; Lean et al., 2005). Other authors have reported no difference in microbial growth when NH_3–N concentrations were below 5 mg/dL (Russell et al., 1983; Bach et al., 2008). In the present study, low NH_3–N concentration observed with CON diets could have been due to a higher use of NH_3–N by starch-degrading microorganisms. Because NH_3–N is the primary N source for cellulose-digesting bacteria, the low concentrations of NH_3–N associated with CON diets could have prevented greater growth of fibrolytic bacteria, resulting in reduced fiber digestion. It is possible that low observed NH_3–N concentrations limited microbial growth; however, this effect was not manifested in a reduction of true OM digestion or efficiency of microbial protein synthesis.

Nitrogen flows (g/d) of NH_3–N and dietary N increased ($P < 0.01$) with BSS inclusion, while daily flows (g/d) of non-NH_3–N and microbial N decreased ($P < 0.01$). Effects of BSS inclusion on nitrogen flows are consistent with findings of Ruiz-Moreno et al. (2015). Nitrogen flow data clearly indicate that BSS inclusion in the diet negatively affected nitrogen metabolism of ruminal microbes. Dietary CP degradation was lower ($P < 0.01$) with BSS inclusion compared with control diets (38 *vs.* 49% of CP intake). Efficiency of microbial protein synthesis expressed as g of microbial N/kg of OM truly digested decreased ($P < 0.01$) with inclusion of BSS, indicating that ruminal microbes were not as efficient in incorporation of available nitrogen into biomass, which is inconsistent with the observations of Ruiz-Moreno et al. (2015), likely due to differences between diet composition and fermentability. Overall, nitrogen metabolism data indicate that BSS inclusion decreased crude protein degradation and reduced microbial protein synthesis.

Amino Acid Flows from Fermenters

Average daily amino acid flows were determined by multiplying measured outflow (g/d) by AA content of the effluent for each fermenter (Table 7). Bismuth subsalicylate reduced ($P < 0.01$) net flow (g/d) for all measured amino acids except Cys, which increased, and Pro, which remained the same as CON. Reduction in AA flow is consistent with the lower EMPS that was observed when BSS was added to fermenters. High dietary sulfur decreased ($P < 0.05$) daily flow of Pro and Cys compared with low sulfur diets. The decrease in flow of Cys with high sulfur treatments is a perplexing result. In the present study, amino acid profile was not determined on the microbial pellet due to insufficient sample amount. Due to large amount of variation in microbial AA flow dependent on diet (Clark et al., 1992) it is difficult to attribute relative changes of specific AA without knowledge of microbial AA profile for each treatment. Relative to dietary intakes of amino acids, all amino acids increased because of the contribution of microbial amino acid synthesis. Reduction in AA flows associated with BSS inclusion can be attributed to the overall negative effects on fermentation that were observed, culminating in a reduction in microbial protein synthesis. Because of the large impact of BSS inclusion on rumen nitrogen metabolism,

Table 6. Effect of bismuth subsalicylate and dietary sulfur level on nitrogen metabolism in continuous culture

	Treatment[1]							
	Low sulfur		High sulfur			*P*-value		
Item	CON	BSS	CON	BSS	SEM[2]	BSS	Sulfur	B × S
NH_3-N, mg/dL	2.7	20.8	2.4	19.9	2.3	< 0.01	0.37	0.67
N flow, g/d								
NH_3-N	0.05	0.40	0.05	0.38	0.04	< 0.01	0.36	0.69
Non NH_3-N	2.10	1.72	2.06	1.67	0.05	< 0.01	0.21	0.88
Microbial-N	1.10	0.50	1.09	0.52	0.08	< 0.01	0.78	0.67
Dietary-N	1.00	1.22	0.97	1.15	0.03	0.02	0.32	0.69
CP degradation, %	47.6	36.2	49.4	40.0	2.4	< 0.01	0.28	0.69
EMPS[3]	29.3	20.6	26.8	20.7	0.8	< 0.01	0.14	0.13

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

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

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

Table 7. Effect of bismuth subsalicylate (BSS) and dietary sulfur level on amino acid flow from fermenters[1]

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

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

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

[3]Standard error of the mean, n = 4 observations per treatment.

it is likely that individual species of bacteria were altered due to relative levels of peptides and NH_3–N. Cotta and Hespell (1986) demonstrated that high levels of amino acids or peptides in ruminal fluid distinctly reduced proteolytic activity of *Butyrivibrio firbrisolvens*. Ruminal microbes have been shown to have varying efficiencies in utilization of AA and peptides depending on availability of energy and nitrogen (Cotta and Russell, 1982; Bach and Stern, 1999). A lower efficiency of AA utilization can be detrimental to achieving high animal production because microbial protein synthesis is not maximized, resulting in an increased need for RUP or expensive protected AA supplements (Chalupa, 1975; Schwab, 1996).

Effluent Fatty Acid Flows from Fermenters

Daily FA intakes and outflows are presented in Table 8. Inclusion of BSS increased ($P < 0.05$) daily FA flows (g/d) likely due to reduced overall DM digestion. Flows of total $C_{18:1}$ *trans* isomers decreased ($P < 0.05$) with BSS inclusion and dietary sulfur level. Flows of $C_{18:0}$ increased in CON diets with high dietary sulfur. Inclusion of BSS reduced flows of *trans*-8, 9, and 10 isomers drastically compared with CON. Total *cis*-$C_{18:1}$ flow increased in CON diets with low dietary sulfur. For individual *cis* isomers, BSS inclusion increased ($P < 0.05$) *cis*-9 and decreased *cis*-11 daily flows.

Cellulolytic bacteria that prefer higher pH such as *Butyrivibrio fibrisolvens* are the main bacteria responsible for biohydrogenation (Harfoot and Hazlewood, 1997). Biohydrogenation of oleic acid can proceed directly to stearic acid, however *cis/trans* isomerization is common by rumen microorganisms (Jenkins et al., 2008). The extent to which this occurs is likely determined by prevailing environmental conditions. In pure culture, Russell and Dombrowski (1980) demonstrated 75% of maximum yield for *Butyrvibrio fibrisolvens* at pH 5.75, however when culture pH was reduced to 5.5, the organism was washed out, possibility due to inability of the organism to attach to substrate at low pH conditions (Martin et al., 2002). In the present study, pH of the CON fermenters was well below 5.5 and the average extent of biohydrogenation of $C_{18:2}$ in CON fermenters (expressed as mg of outflow per 100 mg of input) was 68.7%. This is comparable to Jenkins et al. (2014), who reported average $C_{18:2}$ biohydrogenation of 72%; however, pH in that study averaged 6.0. This indicates that in spite of low culture pH in the present study, biohydrogenation extent remained high. This is in contrast to diets containing BSS, which resulted in low overall *trans* isomerization and high overall $C_{18:2}$ flows, indicating strong negative effects on fatty acid biohydrogenation likely through overall suppression of microbial activity. The influence of sulfur level on fatty acid metabolism in the current study suggest that dietary sulfur

Table 8. Effect of bismuth subsalicylate (BSS) and dietary sulfur level on fatty acid flows from fermenters

	Treatment[1]				
	Low sulfur		High sulfur		
Item	CON	BSS	CON	BSS	SEM[2]
Total fatty acid intake, mg/d[3]	3708.1	3699.0	3697.2	3699.7	-
Individual fatty acid intake, mg/d					
$C_{16:0}$	539.1	537.8	537.5	537.9	-
$C_{18:0}$	66.7	66.5	66.5	66.5	-
$C_{18:1}$	857.1	855.0	854.5	855.1	-
$C_{18:2}$	1855.8	1851.1	1850.2	1851.4	-
$C_{18:3}$	55.9	55.8	55.7	55.8	-
Other	298.9	298.2	298.0	298.2	-
Total fatty acid outflow, mg/d[a,4]	3124.5	3899.9	2904.2	3421.3	183.5
$C_{16:0}$ [a]	476.0	623.3	404.0	499.0	53.2
$C_{18:0}$ [c]	206.8[d]	72.8[d]	396.5[e]	64.0[d]	45.3
$C_{18:1}$ [a,b]	1630.9	943.2	1388.4	862.4	58.3
$C_{18:2}$ [a,b]	617.6	2052.1	550.7	1850.1	59.1
$C_{18:3}$ [a]	16.8	78.5	15.6	56.5	9.2
Other[a,b]	130.4	82.4	111.5	54.8	11.3
Biohydrogenation intermediates					
Total *trans*-$C_{18:1}$ [c]	1026.9[f]	7.2[d]	872.1[e]	9.6[d]	35.4
trans-8[a]	25.8	0.0	30.4	0.0	7.7
trans-9[a]	10.3	0.0	22.5	0.5	6.6
trans-10[a]	981.9	7.2	816.4	8.4	39.7
trans-11	8.9	0.0	2.8	0.8	3.6
Total *cis*-$C_{18:1}$ [a,b]	604.0	936.0	516.3	852.7	37.0
cis-9[a]	552.4	693.4	345.5	828.0	133.8
cis-10	6.2	213.8	131.9	0.0	123.7
cis-11[a]	45.4	28.9	38.9	24.7	4.3
$C_{18:2}$ Conjugated	0.6	0.7	0.2	0.3	0.3

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

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

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

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

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

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

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

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

level plays a role in determining which intermediate isomers are formed, as indicated by total *cis* and *trans* flows. High dietary sulfur also decreased ($P < 0.05$) total $C_{18:1}$ and $C_{18:2}$ flows, and resulted in the highest amount of $C_{18:0}$ flow, indicating possible beneficial effects of sulfur in microbes associated with the biohydrogenation process. Data on dietary sulfur influences on rumen fermentation are limited because many dietary sulfur studies focus on sulfur-associated PEM risks, although some authors reported negative effects on rumen fermentation when dietary sulfur was greater than 0.2% of diet DM (Johnson et al., 1971; Zinn et al., 1997; Spears, 2003). Richter et al. (2012) reported an increase in C18:0 fatty acid concentration in carcasses of steers fed elevated dietary sulfur concentrations, indicating a possible increase in biohydrogenation in the rumen. In general, effects of sulfur on rumen biohydrogenation are not well understood and further investigation may be useful.

Inclusion of BSS at 0.5% of diet DM had strong negative effects on fermentation by ruminal microbes in continuous culture. Digestion, VFA production, N metabolism and fermentation pH data indicate that BSS suppressed overall fermentation. Amount of dietary sulfur (0.21 and 0.42% of diet DM) had no effect on major indicators of fermentation however moderate effects were detected on fatty acid metabolism. Previous research with this compound indicate a strong ability to bind dietary sulfur and reduce toxicity risks, however results from this study indicate that even at 0.5% of diet DM, BSS remains detrimental to rumen fermentation. Future research involving dose-titration in vitro and in vivo are needed to determine whether BSS addition to a ruminant diet can be beneficial.

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