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Effects of Detoxified Sulfur as a Feed Supplement on *in Vitro* Rumen Fermentation and Methane Mitigation

Seon-Ho Kim[1], Mahfuzul Islam[1], Ashraf Ali Biswas[1], Kwang-Keun Cho[2] and Sang-Suk Lee[1]*

[1]*Ruminant Nutrition and Anaerobe Laboratory, Department of Animal Science and Technology, Sunchon National University, Suncheon 57922, Korea*
[2]*Department of Animal Resources Technology, Gyeongnam National University of Science and Technology, Jinju, Gyeongnam 52725, Korea*

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Sulfate is a reductant that competes for electrons and may lower CH_4 production in the rumen. This study was designed to evaluate the beneficial effect of detoxified sulfur powder supplementation on *in vitro* rumen fermentation and methane mitigation. A ruminally cannulated Holstein Friesian cow was used as a rumen fluid source, and commercial pelleted concentrate was used as a substrate at 1 g dry matter. Treatments included the addition of detoxified sulfur powder at the rate of 0% (Control), 0.2% (T1), 0.4% (T2), 0.6% (T3), 0.8% (T4), and 1.0% (T5) as dry matter (DM) basis. The pH, total gas (TG), methane (CH_4) production, DM digestibility, organic matter (OM) digestibility, and volatile fatty acids (VFA) production were analyzed after 12 hr of incubation. The results showed that CH_4 production was significantly lowest in T1 (13.78 ml) but highest in the control (20.16 ml). Insignificantly higher total VFA was observed in control and T1 (64.99 and 64.28 mM, respectively) compared to other treatments after 12 hr of incubation. After 12 hr of incubation, the significantly lowest acetate:propionate was observed in T1 (1.90) while the highest was observed in T4 (2.44). However, no significant differences were recorded for pH, TG, DM digestibility, OM digestibility, acetate, propionate, and butyrate between the control and T1. Total number of bacterial DNA copies was significantly lower in the treatment group than the control. Therefore, it can be concluded from this study that detoxified sulfur at 0.2% inclusion level is optimal for production performance and ruminal CH_4 mitigation.

Key words : Detoxified sulfur, *in vitro*, methane mitigation, ruminant

Introduction

Methane (CH_4) is an important greenhouse gas, that imposes momentous environmental troubles related to global warming. Ruminal methane constitutes up to 15% of global CH_4 emission also contribute a loss of dietary energy (2 to 12%) in ruminants [12]. The average CH_4 emissions are from 161 to 323 g/day in beef cattle and 151 to 497 g/day in dairy cows [4]. Therefore, developing feedings strategies to suppress methane production is deemed necessary. Many factors such as feed intake, feed composition, feed additives, and rumen microflora influence CH_4 emissions from ruminants. The CH_4 emissions can reduce from cattle through manipulation of these factors.

***Corresponding author**
Tel : +82-61-750-3237, Fax : +82-61-750-3237
E-mail : rumen@sunchon.ac.kr

Sulfate is a reductant, reduce to hydrogen sulfide as well as consumes 8 electrons, which compete for the electrons and which may lower CH_4 production [25]. Both synthetic and chemical sulfur can reduce methane production from ruminants [23, 26]. However, excessive dosage (>0.3-0.4%) of dietary sulfur from chemical or synthetic sources either as sulfate or elemental sulfur may lead to toxic effect, even fatal in the extreme case. For a long history of sulfur on the disease producing effect, applying sulfur was limited to animals and humans. The naturally occurring sulfur compounds, as sulfide minerals are mercury sulfide, lead sulfide, zinc sulfide, antimony sulfide, and iron sulfide [20], containing toxic mineral. Detoxification is the process of heating and cooling with water and additives with the repetition of this process in several times. These detoxified compounds can be used as safe feed additives. Thus, researchers are encouraged to develop detoxified sulfur (nontoxic sulfur), which doesn't contain the toxic mineral. Detoxified sulfur can be used as feed additives and influence animal performance [10]. However, detoxified sulfur has not yet tried in *in vitro* rumen fermentation and methane mitigation.

Therefore, the present study was conducted to evaluate the effects of detoxified sulfur on *in vitro* rumen fermentation parameters using pelleted concentrate as a substrate as well as identify the inclusion rate of detoxified sulfur for reducing *in-vitro* CH_4 production.

Materials and Methods

The study was conducted at the animal farms and the Ruminant Nutrition and Anaerobe Laboratory, Department of Animal Science and Technology of Sunchon National University (SCNU), Jeonnam, South Korea. The recommendations of "The Guide for the Care and Use of Laboratory Animals," published by the Institutional Animal Care and Use Committee (IACUC) of the National Institute of Animal Science (2012-C-037) in Korea, were followed during animals' experiment.

In-vitro experiment

An *in vitro* fermentation technique was performed using pelleted concentrate as a substrate. Sulfur powder which has used in this experiment contains 54.42% sulfur and its pH was 8.33. Treatments included 0% (without), 0.2%, 0.4%, 0.6%, 0.8%, and 1.0% of detoxified sulfur as DM basis and, hereafter referred to as Control, T1, T2, T3, T4, and T5, respectively. Ruminal contents were obtained from rumen-cannulated Holstein cow (48-month). The collected ruminal fluid was squeezed and the extracted fluids were strained through 4 layered cheesecloths and were placed in a glass bottle, subsequently capped and immediately transported to the laboratory maintaining the temperature at 39°C. Bottle containing rumen fluid was shaken vigorously by hand before mixing with the buffer. The pooled and particle-free rumen fluid was then transferred to a buffer medium with pH 6.9 [9] at a ratio of 1:3 (rumen fluid: buffer) which was prepared earlier according to Asanuma *et al*. [2]. One hundred milliliter (ml) of buffer mixed rumen fluid was transferred anaerobically, under a constant flow of N_2 gas, into 160 ml serum bottles containing the 1 g pellet substrate (1 mm particle size) (Table 1) and added with detoxified sulfur (Coms. Nazu, Jeonnam, South Korea) at different concentrations. Then, bottles were sealed with rubber septum stopper and aluminum cap [1], and incubated for 12 hr in a shaking incubator with 120 rpm at 39°C [8]. Three replicates and 1 blank per experimental treatment were used in this experiment.

Table 1. Chemical composition of pelleted concentrate feed used for *in vitro* experiment as substrate

Chemical composition	Composition (% of Dry matter)
Dry matter	92.97
Crude protein	14.35
Ether extract	3.69
Crude fiber	7.22
Calcium	1.05
Phosphorus	0.45
Crude ash	6.51
Acid detergent fiber	10.54
Neutral detergent fiber	25.75

Analyses of *in vitro* fermentation parameters

Total gas (TG) production was measured by a pressure sensor meter (EA-6; Sun Bee Instrument Inc., Seoul, Korea) and pH of the fermented rumen fluids were measured by a pH meter (Pinnacle series M530p; Schott Instruments, Mainz, Germany) at 12 hr incubation time point. One aliquot of 1 ml samples from each serum bottle were collected and centrifuged at 13,000× *g* for 10 min at 4℃ (Micro 17TR centrifuge; Hanil Science Industrial Co. Ltd., Gimpo, Korea). The obtained supernatants were collected into new 1.5 ml microtubes and stored at -80°C until volatile fatty acid (VFA) analysis. A separate aliquot of 1 ml samples from each serum bottle was collected and stored at -80°C until microbial population analysis. The gas produced inside the bottle was sampled and trapped inside a vacuum tube for CH_4 determination.

The VFAs were measured according to the methods stated by Han *et al*. [7] and Tabaru *et al*. [24] using high-performance liquid chromatography (HPLC; Agilent Technologies 1200 series, Waldbronn, Baden-Wurttemberg, Germany). The CH_4 in the gas samples was measured by using gas chromatography (GC; HP 5890; Agilent Technologies, San Diego, CA, USA) according to the formula described by Ørskov and McDonald [15]. Before the analysis, the vacuum tubes containing the refrigerated gas samples were warmed by stabling it on the GC machine for at least 30 min. The total CH_4 production per bottle was calculated as parts per million (ppm) multiplied by the TG produced [27].

Determination of *in vitro* dry matter and organic matter digestibility

Before starting the *in vitro* rumen fermentation, Dry matter (DM) and organic matter (OM) content of the pellet substrate were analyzed by drying at 105℃ for 16 hr and ashing

at 550℃ for 12 hr, respectively. The percents of DM and OM before incubation were used as the initial DM (DM_i) and initial OM (OM_i), respectively for calculation. After 12 hr incubation, the fermented samples from each serum bottle were poured in dried and pre-weighed nylon bags and knotted using nylon thread, then splashed with water flow for 15 minutes or until the disappearance of turbidity of washed water. The final DM (DM_f) and OM (OM_f) of the substrate were determined by the same protocol followed for the initial values (DM_i and OM_i). The DM and OM digestibility (%) were calculated as ({[$DM_i - DM_f$]/DM_i} ×100) and ({[$OM_i - OM_f$]/OM_i} ×100), respectively.

Quantitative real-time PCR (qPCR)

Total bacteria, protozoa, methanogens, *Fibrobacter succinogenes*, *Ruminococcus flavefaciens* and fungal DNA copies were estimated by using the protocol of Christophersen [5]. The concentration of gDNA extracted from each sample was determined by using Epoch Microplate Spectrophotometer (Biotek^R). PCR forward and reverse primers were used for an individual organism. Total bacterial primers Bac 1114F (5′-CGG CAA CGA GCG CAA CCC-3′) and Bac 1275R (3′-CCA TTG TAG CAC GTG TGT AGC C-5′), methanogens primer qmcraA-F (5′-GGA TTA GAT ACC CSG GTA GT-3′) and qmcrA-R (3′-GTT GAR TCC AAT TAA ACC GCA-5′), protozoal forward primer F (5′-GCT TTC GWT GGT AGT GTA TT-3′) and reverse primer R (3′-CTT GCC CTC YAA TCG TWC T-5 ′), *F. succinogenes* forward primer F (5′-GTT CGG AAT TAC TGG GCG TAA A-3′) and reverse primer R (3′-CGC CTG CCC CTG AAC TAT C-5′), *R. flavefaciens* forward primer F (5′-CGA ACG GAG ATA ATI TGA GTI TAC TTA GG-3′) and reverse primer R (3′-CGC TCT CTG TAT GTT ATG AGG TAT TAC C-5′), fungal forward primer F (5′-GAG GAA GTA AAA GTC GTA ACA AGG TTT C-3′) and reverse primer R (3′-CAA ATT CAC AAA GGG TAG GAT GAT T-5′) were used. Amplification was done by Eco Real-Time PCR (Illumina, Inc) with a total volume of 20 μl per reaction mixture (10 μl of 2x qPCRBIO SyGreen Mix, 0.8 μl each forward and reverse PCR primers, and 8.4 μl template DNA of 50 ng/μl in sterile distilled water).

Statistical analysis

All the data were analyzed using the general linear model (GLM) under a completely randomized design. The significant (p<0.05) differences between and among treatments and control were identified by Duncan's Multiple Range Test and Orthogonal Polynomial Contrast. The Statistical Analysis System (SAS) (version 9.1; SAS Inst. Inc., Cary, NC) was used to carry out the above analysis [21].

Results

In vitro fermentation, Methane (CH_4) production, and digestibility

The pH, TG, acetate, and total VFA were not different among treatments (p>0.05) (Table 2). However, a significant difference (p<0.05) was observed on propionate. The propionate was significantly highest in control (18.81 mM) and lowest in T3 and T4 (15.32 and 14.20 mM) after 12 hr of incubation. In case of butyrate, significant difference (p<0.05) was observed after 12 hr of incubation which the highest value was in T5 (13.23 mM) and the lowest was in control

Table 2. The pH, total gas and Volatile Fatty Acid production from *in vitro* rumen fermentation by the addition of sulfur

Parameters	Treatments						SEM	P value	
	Control	T1	T2	T3	T4	T5		All	C. vs T.
pH	5.29	5.26	5.30	5.26	5.23	5.25	0.016	0.154	0.173
Total gas (ml/g of DM)	64.50	65.00	65.33	65.33	63.67	62.67	0.972	0.465	0.944
DM digestibility (%)	55.00	56.75	55.50	53.50	54.80	58.00	1.165	0.435	0.690
OM digestibility (%)	53.25	50.75	51.00	50.25	48.00	54.00	1.691	0.336	0.275
Acetate, mM	34.45	35.37	34.51	34.40	34.57	34.60	0.257	0.252	0.461
Propionate, mM	18.81^{a}	16.95^{ab}	16.96^{ab}	15.32^{b}	14.20^{b}	16.04^{ab}	0.629	0.039	0.010
Butyrate, mM	11.74^{c}	11.96^{bc}	11.83^{bc}	12.67^{abc}	12.93^{ab}	13.23^{a}	0.324	0.038	0.058
Total VFA, mM	64.99	64.28	63.04	62.39	61.71	63.87	0.797	0.311	0.183
Acetate: Propionate	1.83^{d}	1.90^{cd}	2.14^{bc}	2.26^{ab}	2.44^{a}	2.17^{abc}	0.070	0.003	0.002

DM: Dry matter, OM: Organic matter.
Control: Without sulfur, T1: 0.2% sulfur, T2:0.4% sulfur, T3:0.6% sulfur, T4:0.8% sulfur, T5:1.0% sulfur.
C. vs T. is the contrast between control and treatment.
^{a,b,c} Means with different superscripts in the same row are significantly different (p<0.05).

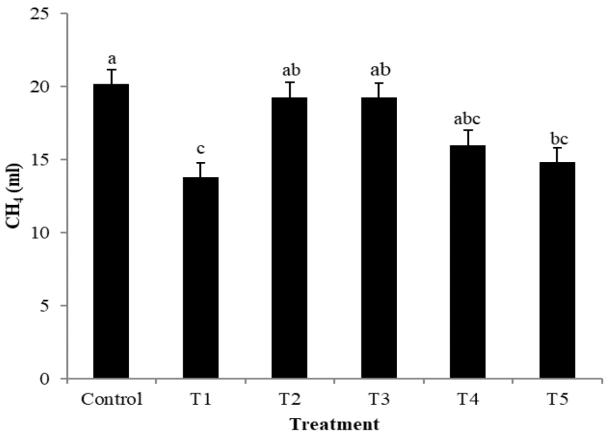

Fig. 1. Methane production from *in vitro* rumen fermentation after 12 hr incubation by the addition of Sulfur. Control: Without Sulfur; T1: 0.2% Sulfur; T2:0.4% Sulfur; T3:0.6% Sulfur; T4:0.8% Sulfur; T5:1.0% Sulfur; [a,b,c] Means with different superscripts in the same row are significantly different (p<0.05).

(11.74 mM). There was a significant difference in acetate: propionate (A: P) value was observed after 12 hr of incubation. Moreover, the lowest A: P value was observed in control (1.83) followed by T1 (1.90), and the highest was in T4 (2.44) treatment. After 12 hr of incubation, the lowest CH_4 production was observed in T1 (13.78 ml) followed by T5 (14.81 ml) and the highest in the Control (20.16 ml) (Fig. 1). The DM and OM digestibility were not varied significantly among control and treatments (p>0.05) (Table 2).

Microbial DNA copies by qPCR

The result of total bacteria from the qPCR revealed that there was a significant difference (p<0.05) between control and treatments. The highest (DNA copies) value of total bacteria were observed in control (9.27×10^8) compared to treatments where T1 was 3.51×10^8, T2 was 3.86×10^8, T3 was 3.38×10^7, T4 was 1.83×10^8 and T5 was 3.09×10^8(Table 3). Protozoal DNA copies were significantly different (p<0.05) among control and treatments where the lowest DNA copies were in T4 (4.47×10^4) within the treatments. There was no significant difference in the case of methanogens DNA copies, but numerically lower methanogen was observed in treatment groups than the control. Higher methanogen (DNA copies) were in control (1.04×10^6) than the treatment group which were 9.13×10^5, 4.82×10^5, 4.96×10^5, 2.16×10^5, 2.50×10^5 in T1, T2. T3, T4, and T5, respectively. *Fibrobacter succinogenes* DNA copies were significantly different (p<0.05) among treatments and control group. Higher DNA copies were observed in control and T1 (5.96×10^5 and 5.40×10^5) than in T2, T3 and T4 (3.00×10^5, 2.88×10^5 and 1.71×10^5, respectively). Likewise, *Ruminococcus flavefaciens* DNA copies were significantly different (p<0.05) among the treatments and control groups. Higher DNA copies were observed in T4 (7.67×10^4) than the others treatments and control groups (p<0.05). There was no significant difference in fungal DNA copies among the control and treatments group.

Discussion

Feed additives play a significant role in rumen fermentation as well as CH_4 emission. The CH_4, a greenhouse gas, contributes 20-50 times more than that of CO_2 to exert the greenhouse effect [3, 6]. Sulfur can be used to improve rumen fermentation as well as methane mitigation in ruminants as a feed additive. The NRC [14] suggested that the maximum tolerable amount of sulfur is 0.3% (DM basis) for beef cattle with high-concentrate diets and 0.5% for beef cattle with high forage diets. Moreover, researchers are struggled to find out the optimum dose of sulfur for a ruminant.

In the current experiment, CH_4 production was signifi-

Table 3. Microbial DNA copies by qPCR after 12 hr incubation from *in vitro* fermentation by the addition of sulfur

Experimental microbes	Treatments						SEM	P value	
	Control	T1	T2	T3	T4	T5		All	C. vs T.
Total bacteria	9.27×10^{8a}	3.52×10^{8b}	3.87×10^{8b}	3.39×10^{8b}	1.83×10^{8b}	3.09×10^{8b}	1.09×10^8	0.044	0.003
Protozoa	7.38×10^{4abc}	7.63×10^{4ab}	8.56×10^{4a}	4.79×10^{4bc}	4.47×10^{4c}	5.13×10^{4bc}	7.89×10^4	0.045	0.235
Methanogens	1.04×10^6	9.13×10^5	4.82×10^5	4.96×10^5	2.16×10^5	2.50×10^5	1.96×10^5	0.279	0.108
F. succinogenes	5.96×10^{5a}	5.40×10^{5a}	3.00×10^{5bc}	2.88×10^{5bc}	1.71×10^{5c}	4.56×10^{5ab}	4.77×10^4	0.001	0.001
R. flavefaciens	1.86×10^{4b}	2.03×10^{4b}	1.10×10^{4b}	7.84×10^{3b}	7.67×10^{4a}	1.15×10^{4b}	4.04×10^3	0.001	0.332
Fungi	2.65×10^3	4.06×10^3	4.52×10^3	3.10×10^3	1.59×10^3	4.57×10^3	6.53×10^2	0.274	0.335

Control: Without sulfur, T1: 0.2% sulfur, T2:0.4% sulfur, T3:0.6% sulfur, T4:0.8% sulfur, T5:1.0% sulfur.
C. vs T. is the contrast between control and treatment.
[a,b,c] Means with different superscripts in the same row are significantly different (p<0.05).

cantly lowest at 0.2% detoxified sulfur supplemented group. Lower CH_4 production noted with 0.2% sulfur supplementation agrees with the findings of Pakmaluek *et al*. [16], who stated 0.2% sulfur supplementation significantly lower CH_4 production while using rice straw as substrate. This result also supported by the Ungerfield and Kohn [25] who revealed that sulfate reduction is more favorable than methanogenesis. Isa *et al*. [11] confirmed that low sulfate concentration direct relatively more electrons toward sulfate reduction compare with a high sulfate concentration.

The present study of the experiment stated that there was no significant difference in pH after 12 hr of incubation period that was supported by Kung *et al*. [13]. After 12 hr of incubation, the lack of effect of sulfur on total gas is consistent with the findings of Smith *et al*. [22] and Kung *et al*. [13], who also reported non-significant TG production with sulfur supplementation. The non-significant acetate concentration observed on our study is similar to the findings of Kung *et al*. [13], who described lack of effect of sulfur concentration at a rate of 0.29% and 1.09% (DM basis) on VFA production with a medium-concentrate substrate *in vitro*. Rahman *et al*. [19] revealed similar findings when sodium lauryl sulphate, fumaric acid, and their coupled addition were used as treatments. Significantly lower propionate concentration was observed in treatments than control in our experiment. The result of this experiment is tended to similar to the findings of Promkot *et al*. [17], where they reported that lower propionate concentration was recorded along with the increasing of sulfur supplementation.

After 12 hr of incubation, the lack of effect of sulfur on DM and OM digestibility, observed in this study, is consistent with the findings of Quinn *et al*. [18], who stated the DM digestibility was not differed with sulfur supplementation at 0.17 or 0.42% of DM. Similarly, Zinn *et al*. [28] revealed no effects of sulfur supplementation in diet ranging from 0.15% to 0.25% (DM basis) on OM digestibility *in vivo*. In the current study, significantly ($p<0.05$) lower total bacterial DNA copies and tended to lower methanogens DNA copies were revealed in the treatment groups than the control, which is related to the CH_4 reducing tendency in sulfur supplemented group.

In conclusion, detoxified sulfur at 0.2% inclusion level is competent to decrease CH_4 production. Consequently, detoxified sulfur at lower inclusion level (0.2% DM) is a prospective feed supplement for ruminal CH_4 mitigation and increasing productivity of ruminant animal.

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The Conflict of Interest Statement

The authors declare that they have no conflicts of interest with the contents of this article.

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초록 : 제독 유황의 반추위 발효성상 및 메탄 저감 효과 연구

김선호[1] · 이스람 마푸줄[1] · 비스와스 아쉬라프 알리[1] · 조광근[2] · 이상석[1]*
([1]순천대학교 동물자원과학과 반추영양 및 혐기미생물연구실, [2]경남과학기술대학교 동물소재공학과)

제독유황은 황산염의 환원제로서 황화수소로 환원되면서 메탄생성과정에서 에너지 이용 경쟁을 통해 저감 할 수 있다. 본 연구는 유독성분이 제거된 유황을 *in vitro*를 통해 반추위 발효 성상 및 메탄 저감 효과를 평가하였다. 반추 위액은 케뉼라가 부착되어 있는 홀스타인 젖소에서 채취하여 이용하였으며, 기질 사료는 농후사료 1 g (DM)을 사용하였다. 처리구는 0% (대조구), 0.2% (처리구 1), 0.4% (처리구 2), 0.6% (처리구 3), 0.8% (처리구 4) 및 1.0% (처리구 5)로 실시하였다. pH, 총가스발생량 (TG), 메탄, 건물 소화율, 유기물 소화율 및 휘발성지방산을 분석하였다. 메탄생성량은 0.2% 처리구에서(13.78 ml) 가장 낮았으며, 대조구에서(20.16 ml)로 가장 높았다(p<0.05). 휘발성지방산은 0.2% 처리구에서 64.28 mM 높았다. A/P 비율은 0.2% 처리구에서 1.90으로 가장 낮았으며, 0.8%에서 2.44로 유의성이 있었지만, pH, 총가스발생량, DM 소화율, OM 소화율, acetate, propionate 및 butyrate에서는 차이가 없었다. 총 세균의 DNA는 대조구보다 모든 처리구에서 낮았다. 이 연구를 통해 0.2% 처리구에서 반추위 발효 성상 및 메탄 저감에 효과가 있었다.