Effects of reductive acetogenic bacteria and lauric acid on in vivo ruminal fermentation, microbial populations, and methane mitigation in Hanwoo steers in South Korea

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ABSTRACT: Animal science nutrition studies are increasingly focusing on finding solutions to reduce methane (CH₄) emissions. In the present study, we evaluated the effect of reductive acetogenic bacteria [acetogen probiotics (AP)] and lauric acid (LA) on in vivo rumen fermentation and microbial populations in Hanwoo steers. Four cannulated Hanwoo steers (392 ± 14 kg) were analyzed in a 4 × 4 Latin square design and were placed in hood-type chambers. They were fed similar amounts of concentrate and rice straw within and experimental design as follows: control (Con; 40 g DM basal feed, nonaddition of AP or LA), T1 = LA (40 g DM basal feed mixed with 40 g LA), T2 = AP (40 g DM basal feed, fermented with AP), and T3 = LA + AP (40 g DM basal feed, fermented with AP and mixed with 40 g LA). The animals were acclimatized to the diet for 15 d, followed by 6 d of the experimental period. Rumen fluid samples for metabolite and molecular analyses were collected 6 h after the morning feeding, with 2-h collection intervals. The enteric CH₄ production was monitored on the last 2 d of

the experimental period. Concentrations of total volatile fatty acids increased with the increase in time after feeding. Acetate, propionate, and butyrate concentrations were observed to be higher in the treatments than in Con. The addition of LA and AP reduced CH₄ emission compared with that of Con ($P < 0.01$). Nuclear magnetic resonance spectroscopy results revealed no correlation between the LA and Con groups, but AP showed a correlation with LA and Con. Reduction in the number of protozoa which was accompanied by a decrease, because methanogens live symbiotically with protozoa. Supplementation of AP or LA alone and in combination decreased ($P < 0.05$) the methanogen population, whereas supplementation of LA alone significantly increased ($P < 0.05$) *Ruminococcus flavefaciens* and slightly increased total fungi. Thus, dietary supplementation of LA and AP has inhibitory effects on CH₄ production in Hanwoo cattle. If the effects of this method can be maintained, reductive acetogens could become an important part of strategies to lower ruminant CH₄ emissions.

Key words: acetogen, in vivo, lauric acid, methane

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INTRODUCTION

Methane, an important greenhouse gas, imposes significant ecological problems related to global warming. Several animal science nutrition

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studies are attempting to obtain solutions to reduce methane (CH₄) emissions. The abatement of CH₄ emissions from ruminants might be achieved through different strategies such as feed manipulation; defaunation; vaccination; and supplementation with lipids, organic acids, plant compounds, microbes, or chemical additives (Hook et al., 2010). Saturated medium-chain fatty acids (MCFAs) have a high potential to suppressing ruminal methanogenesis. Blaxter and Czerkawski (1966) demonstrated in vivo that lauric acid (C₁₂; LA) can suppress total tract CH₄ production at a reduction rate of 68%. Another potential strategy to reduce ruminant greenhouse gas emissions is through microbial supplementation. Among these microbes are the acetogens, which were discovered as early as 1932 (Fischer et al., 1932). Acetogens undergo reductive acetogenesis, which is an alternative hydrogen-utilizing pathway that may serve as a hydrogen sink in decreasing CH₄ emissions (Van Nevel and Demeyer, 1995; Joblin, 1999). Homoacetogens use the acetyl-CoA pathway to reduce carbon dioxide to acetate, and most are able to use this pathway for growth using hydrogen and carbon dioxide as the sole energy sources (Drake et al., 2008).

We recently isolated and identified an acetogenic bacterium closely related to *Proteiniphilum acetatigenes* (GA03) from the Korean native goat (*Capra hircus coreanae*). This acetogenic bacterium is closely related to the bacterial strain *P. acetatigenes* TB107^T, reported by Chen and Dong (2005). *P. acetatigenes* TB107^T mainly produces acetic acid from fermentation and is also capable of producing propionic acid from yeast extract (Chen and Dong, 2005). Hence, we investigated the effect of our isolated acetogenic bacterium (GA03) and a saturated fatty acid, LA, on in vivo fermentation parameters, with an emphasis on mitigating CH₄ production.

MATERIALS AND METHODS

Animals and Experimental Design

The experimental procedures involving the use of animals were approved by the animal care and use committee at the Rural Development Administration, and the study was conducted at the Animal Environment Division research farm, National Institute of Animal Science, Suwon, South Korea. Four ruminally cannulated Hanwoo steers (392 ± 14 kg) were used in a 4 × 4 Latin square design for 21-d experimental periods. The steers were kept in a hood-type chamber system

with 1 steer per chamber. For the entire experiment, the feed intake was restricted to 5 kg of concentrate feed (DM) and 1 kg of rice straw (DM) per animal twice a day. The nutrient composition of feed materials fed to the animals is shown in Table 1. The basal feed used in the in vivo experiment was soybean meal (Woojung Trading, Jeonnam, South Korea) and wheat bran (Woojung Trading, Jeonnam, South Korea) (1:1 ratio). Acetogen probiotics (AP) comprised GA03. For treatments with AP, the basal feed was inoculated with 10% AP and incubated for 3 d with 4.02 log cfu/g. The LA (dodecanoic acid) used was purchased from Sigma-Aldrich (>98% concentration), which was from *Cocos nucifera*. The experimental designs were as follows: control (40 g DM basal feed, nonaddition of AP or LA), T1 = LA (40 g DM basal feed mixed with 40 g LA), T2 = AP (40 g DM basal feed, fermented with AP), and T3 = LA + AP (40 g DM basal feed, fermented with AP and mixed with 40 g LA). The animals were acclimatized to the diet for 15 d, followed by 6 d of the experimental period.

Analyses of In Vivo Parameters and Metabolite Profiling

Rumen fluids were collected 6 h after the morning feeding, with 2-h collection intervals. The pH was determined using the Pinnacle series M530p meter (Schott Instruments D-55122, Mainz, Germany), whereas the concentrations of VFAs were analyzed using gas chromatography (VARIANCP-3800; USA) using the method described by Erwin et al. (1961). Five milliliters of filtered rumen fluid was added to 1 mL of 25% metaphosphoric acid (wt/vol) and then stored at -20 °C until analysis.

One milliliter of each of the respective supernatants obtained by centrifugation was lyophilized and dissolved in 99.9% deuterium oxide (600 µL of D₂O) with 5 mM sodium 2,2-dimethyl-2-silapentane-5-sulfonate (97%) and then centrifuged at 13,000 rpm for 5 min. The resulting supernatants

Table 1. Nutrient composition of ration fed to the animals in the in vivo experiment (percent DM)

Parameters	Rice straw	Concentrate mixture
Parts of feed offered (%)	17	83
Nutrient composition		
DM, %	88.73	91.90
CP, %	4.54	13.30
EE ¹ , %	0.81	3.35
Crude fiber, %	33.33	4.55
Crude ash, %	11.47	4.88

¹EE = ether extract.

were transferred into 5-mm nuclear magnetic resonance (NMR) tubes, and their NMR spectra were acquired using a Varian Inova 600 MHz NMR spectrometer (USA). Rumen metabolites were identified and quantified on the basis of the NMR spectra using the Chenomx NMRsuite program (v6.1; Chenomx, Canada) as described by [Ametaj et al. \(2010\)](#) and [Jung et al. \(2011\)](#). Briefly, all NMR spectra for multivariate statistical analysis were phased and baseline corrected by MestReNova v5.2.5 software (Spain). The NMR spectral data of 0.5 to 10.0 ppm were reduced into 0.04 ppm spectral buckets and normalized to the total spectral area. The NMR spectra were converted to the ASCII format, and the region of 4.6 to 4.8 ppm, corresponding to water, was removed. The resulting ASCII format files were imported into the MATLAB program and mean centered with no scaling. Principal component analysis (PCA) was performed using MATLAB PLS_Toolbox (v3.5; Eigenvector Research, USA) at a confidence level of 95%.

Respiration Chamber (Hood-Type Chambers)

On the last 2 d of the experimental period for each sampling period within the treatment period, Hanwoo steers were divided into 4 hood-type respiration chambers. Sealing prevented leakage of the internal air system for efficient analysis of O₂, CO₂, and CH₄. The framework dimensions of the metabolic chamber were 230 cm (L) × 140 cm (H) × 160 cm (W), and 115 cm (L) × 100 cm (H) × 140 cm (W). The chambers were constructed in a way that would allow easy monitoring.

Measurement of Changes in Rumen Microbial Distribution

The method described by [Denman et al. \(2007\)](#) was used for measuring changes in the rumen microorganisms. The reactions were performed with a real-time PCR instrument (Corbett Rotor-Gene 6000; Bio-Rad, USA) using the SYBR Green Realtime PCR Master Mix (Code No. QPK-201). Polymerase chain reaction amplification conditions were as follows: 50 °C for 2 min, initial denaturation of 2 min at 95 °C, and 40 cycles of 95 °C for 15 s and 60 °C for 60 s. Quantitative analysis of the relative gene expression was carried out by calculating the relative Ct values. The primer sequences and procedure used were according to [Denman and McSweeney \(2006\)](#) and [Hook et al. \(2009\)](#).

Statistical Methods

Data were carried out in Statistical Analysis Systems (SAS) version 9.1 ([SAS Institute, 2012](#)). The effect of the different treatments was assessed by the general model for a Latin square design. Duncan's multiple range test was used to identify differences between specific treatments; $P < 0.05$ was considered statistically significant.

RESULTS

All the feed given to the animals was consumed; hence, no refusal was recorded in this study. The pH values were comparable among treatments and were the highest in the AP-supplemented diet at all times and the lowest in the LA-supplemented diet at 4 h after morning feeding ([Table 2](#)). A decline in pH was also observed from 0 to 4 h after feeding, increasing slightly after 6 h. As also shown in [Table 2](#), the amount of total VFAs in the LA-supplemented diet had the highest ($P < 0.01$) concentration at 6 h after feeding (101.21 mM), whereas the control (58.31 mM), T2 (61.81 mM), and T3 (61.92 mM) concentrations showed no significant differences. Acetate and propionate concentrations were also high ($P < 0.05$) in LA-supplemented animals 6 h after feeding (62.62 and 29.43 mM, respectively). The combination of LA and AP in the diet increased the total VFAs, as well as acetate and propionate, 4 h after morning feeding. Meanwhile, the A:P ratio at 6 h after feeding the T3 diet was lower than that of other treatments. [Figure 1](#) shows the PCA using the average of the NMR spectra. The graph showed no correlation between the LA and Con groups, but AP was correlated with LA and Con.

As shown in [Fig. 2](#), T2 decreased CH₄ production slightly, whereas T1 and T3 highly significantly ($P < 0.01$) reduced CH₄ production. The supplementation of LA + AP led to the lowest total CH₄ emissions (20.23 L·d⁻¹·kg⁻¹ of DMI) and significantly differed ($P < 0.01$) from that of the control (27.32 L·d⁻¹·kg⁻¹ of DMI). [Table 3](#) shows the microbial profile of ruminal fluid collected from Hanwoo steers of different treatments, particularly of fungi, cellulolytic bacteria (*Fibrobacter succinogenes* and *Ruminococcus flavefaciens*), and methanogens, at 6 h after feeding. Supplementation with AP alone significantly reduced ($P < 0.05$) the methanogen population, followed by supplementation with LA alone, and LA + AP (log cfu values of 5.23, 10.19, and 11.48, respectively). The *F. succinogenes* population was significantly decreased ($P < 0.05$) by

Table 2. Effect of different treatments on fermentation characteristics in the rumen

Parameters	Fermentation time (h)	Treatment				SEM
		Control	LA ¹	AP ²	LA + AP ³	
pH	0	6.90	7.06	7.01	7.09	0.53
	2	6.54	6.55	6.60	6.63	0.74
	4	6.34	6.18	6.46	6.39	0.33
	6	6.43	6.41	6.57	6.42	0.80
Total VFA, mM	0	40.02	32.48	28.57	25.87	4.88
	2	56.64	65.21	39.06	58.80	5.84
	4	79.40	64.95	69.00	81.76	0.98
	6	58.31 ^b	101.21 ^a	61.81 ^b	61.92 ^b	0.84
Acetate, mM	0	27.78	22.21	19.24	18.00	1.99
	2	39.34	43.89	25.23	39.34	4.21
	4	53.30	36.64	42.98	54.76	5.59
	6	36.16 ^b	62.62 ^a	38.85 ^b	35.97 ^b	3.78
Propionate, mM	0	6.91	6.61	4.78	5.26	0.54
	2	10.88	13.84	8.79	13.37	1.15
	4	16.81	18.87	15.98	19.57	1.87
	6	13.62 ^b	29.43 ^a	13.67 ^b	17.93 ^b	1.42
Butyrate, mM	0	5.33	3.66	4.55	2.61	0.39
	2	6.42	7.48	5.04	6.09	0.66
	4	9.29	9.44	10.04	7.43	1.10
	6	8.53	9.16	9.29	8.20	0.86
A:P ratio	0	3.72	3.50	3.89	3.40	0.92
	2	3.47	2.98	2.99	5.23	0.54
	4	3.07	2.22	2.80	2.65	0.18
	6	2.87	2.89	2.91	2.13	0.55

Values are means of triplicates; means with different superscripts (^{a,b}) among treatments are significantly different ($P < 0.05$).

¹Lauric acid.

²Acetogen probiotics.

³Lauric acid + acetogen probiotics.

supplementation with AP and LA alone or combined. Notably, LA supplementation significantly ($P < 0.05$) increased *R. flavefaciens* and slightly increased total fungus.

DISCUSSION

Saturated MCFAs are known to have a high potential in suppressing ruminal methanogenesis, whereas acetogenic bacteria compete with methanogens by utilizing H_2 and CO_2 as substrates to form acetic acid and water. Through this pathway, CH_4 production is reduced. The tendency of the AP-supplemented diet to increase pH corresponds with the pH values of 6 reductive acetogenic bacteria added to rumen fluid incubated in vitro (Lopez et al., 1999), whereas the lowest pH value in the LA-supplemented diet in this study was comparable to the observation of Dohme et al. (2001) in rumen fermentation supplemented with LA using the rumen simulation technique. However, LA had a higher pH value than did other MCFAs, stearic acid, and myristic acid (MA), when given to lactating dairy cows (Hristov et al., 2011).

The fermentation of feed in the rumen by bacteria, protozoa, and fungi produces H_2 , which is used by some bacteria and methanogens to obtain energy, while generating metabolic end products such as VFAs through this process (Martinez-Fernandez et al., 2016). LA-supplemented and control groups in the study by Dohme et al. (2001) had comparable concentrations during rumen fermentation using the rumen simulation technique. In addition, VFA concentrations were not affected by exogenous acetogens (Yang et al., 2015) or by 6 reductive acetogenic bacteria from a variety of ruminal and nonruminal environments (Lopez et al., 1999) when added in in vitro rumen fermentation, which corroborates the findings of this study when acetogen was independently provided to the animals. Meanwhile, the H-NMR spectroscopy results revealed no significant difference among treatments and no correlation between the LA and Con groups. AP was found to be correlated with LA and Con, indicating that LA addition greatly affects metabolite production by microorganisms in the rumen. Furthermore, differences in metabolite production might be due to the addition of

Figure 1. Nuclear magnetic resonance spectrum of the average of each period of rumen metabolites using principal component analysis. LA = lauric acid, acetogen = acetogen probiotics, LA + Ace = lauric acid + acetogen probiotics.

Figure 2. Effect of refined LA and AP supplementation on CH₄ output (L·head⁻¹·d⁻¹). Values are means of triplicates $\pm$ SE. Means with different superscripts (^{a-c}) among treatments are significantly different ($P < 0.05$). LA = lauric acid, AP = acetogen probiotics, LA + AP = lauric acid + AP acetogen probiotics.

feed additives, which alters the rumen microbial diversity and population. Lee et al. (2012) showed a relatively clear separation of rumen samples on the basis of microbial diversity, which was the opposite of the result obtained among treatments in this study. This might be due to differences in the samples used in the study; Lee et al. (2012) investigated metabolites of ruminant species and breeds with different feeding diets and raised at different locations, whereas in this experiment, we used a single breed, Hanwoo cattle.

Methanogenic archaea are the main consumers of the H₂ in this ecosystem, producing CH₄ as the end product (Ungerfeld and Kohn, 2006). Losses from CH₄ produced by enteric fermentation in ruminants can vary by as much as 2% to 12% of the ingested gross energy by the animal (Johnson and Johnson, 1995). A hood-type chamber enables the measurement of the CH₄ output released from the oronasal opening but not the CH₄ excreted from the anus. As described by Murray et al. (1976), rumen production accounts for 87% of the total CH₄ production, from which 95% is excreted via eructation, whereas 89% and 11% of CH₄ in the lower digestive tract is emitted through the lungs and anus, respectively. Blaxter and Czerkawski (1966) demonstrated in vivo that LA (C₁₂) is more potent in suppressing total tract CH₄ production than MA (C₁₄), at 68% and 49% reduction rates, respectively. Soliva et al. (2003, 2004) obtained the same results with combined LA and AP, such that their synergistic action resulted in more efficient CH₄ production abatement than when either is used alone. Moreover, Soliva et al. (2003) reported that the CH₄-suppressing effect of the MCFA mixtures depended mainly on the amount of LA added. Furthermore, supplementing ruminant diets with coconut oil, containing high amounts of LA and MA, decreased CH₄ production in vitro by up to 88% (Dong et al., 1997) and in vivo by up to 73% (Machmüller and Kreuzer, 1999).

Table 3. Effects of different treatments on the rumen microbial population (6 h)

Target microbes (log cfu)	Treatments				SEM
	Control	LA ¹	AP ²	LA + AP ³	
Total fungi (10 ³)	7.98 ^{ab}	9.99 ^a	3.23 ^b	6.50 ^{ab}	<0.01
<i>Fibrobacter succinogenes</i> (10 ²)	6.06 ^a	1.93 ^b	1.08 ^b	2.40 ^b	0.01
<i>Ruminococcus flavefaciens</i> (10 ³)	7.23 ^b	43.14 ^a	6.36 ^b	42.57 ^a	0.02
Methanogens (10 ²)	28.13 ^a	10.19 ^b	5.23 ^c	11.48 ^b	0.05

Values are means of triplicates; means with different superscripts (^{a,b}) among treatments are significantly different ($P < 0.05$).

¹Lauric acid.

²Acetogen probiotics.

³Lauric acid + acetogen probiotics.

The decreasing tendency of CH₄ production in AP supplementation indicates that AP can provide an alternative H₂ sink, which is an essential intermediate in CH₄ formation. This decreasing tendency of CH₄ corroborates the findings of Joblin (1999) that CH₄ production decreased in vitro in the presence of a rumen acetogen isolate and the methanogen *Methanobrevibacter smithii*, isolated from a grazing sheep. In addition, Lopez et al. (1999) found that only 2 of 6 reductive acetogens, *Eubacterium limosum* ATCC 8486 and acetogenic bacteria Ser 5, decreased CH₄ production. Fonty et al. (2007) also reported that reductive acetogenesis was not as efficient as methanogenesis as a H₂ sink in the rumen, and hence, in this experiment, reductive acetogenesis was found to only decrease CH₄ production.

The reduction in the number of protozoa is accompanied with a decrease in the methanogen population, given that methanogens live symbiotically with protozoa. Newbold et al. (1995) reported that methanogens might also be associated with rumen ciliate protozoa, and Matsumoto et al. (1991) demonstrated in vivo the particular sensitivity of rumen ciliate protozoa to LA and MA. In addition, reduced protozoal counts in LA-supplemented groups were comparable to those in studies by Hristov et al. (2011) and Faciola and Broderick (2013). Supplementation of AP significantly reduced the methanogen population, which implies that AP might have competed with methanogen in H₂ consumption. This result supports the tendency to decrease CH₄ concentration in AP alone and significantly decrease the CH₄ concentration in the combined LA + AP supplementation. Several researchers (Genthner et al., 1981; Greening and Leedle, 1989; Morvan et al., 1994) have isolated bacteria from the rumen, which perform reductive acetogenesis; however, they are few in number, and attempts to increase acetogenesis have not been successful, largely because reductive

acetogens cannot compete with the methanogenic archaea under ruminal conditions (Demeyer et al., 1996; Nollet et al., 1997, 1998). Although no significant reduction in CH₄ concentration was observed in this experiment, the direct addition of acetogenic bacteria in feed resulted in a reduction in the methanogen population. Thus, the addition of AP is a feasible alternative to methanogenesis in the ruminal ecosystem.

A decrease in methanogen population was also observed with LA supplementation alone. Soliva et al. (2004) explained that the immediate inhibitory action of LA on ruminal methanogens and CH₄ release occurred with the addition of MA, which enhanced the CH₄-suppressing effect of LA in certain mixtures. Dohme et al. (2001) further explained that supplementation with LA alone has a high CH₄-suppressing effect. Furthermore, a significant increase in the *R. flavefaciens* population indicates that LA supplementation increased the abundance of the cellulolytic bacterium *R. flavefaciens*.

CONCLUSION

Our results showed that LA and reductive AP are favorable if they are established in the rumen and used as methanogen-suppressing agents. This is a safe and reliable method for eliminating or inhibiting ruminal methanogens. Further research and development of the method might enable the maintenance of these effects on a larger scale, and reductive acetogens might become an integral part of strategies to lower ruminant CH₄ emissions.

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