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Fumarate Reductase-Producing Enterococci Reduce Methane Production in Rumen Fermentation In Vitro

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Biotic agents such as fumarate-reducing bacteria can be used for controlling methane (CH_4) production in the rumen. Fumarate-reducing bacteria convert fumarate to succinate by fumarate reductase, ultimately leading to the production of propionate. Fumarate-reducing bacteria in the genus *Enterococcus* were isolated from rumen fluid samples from slaughtered Korean native goats. The enterococci were identified as *Enterococcus faecalis* SROD5 and *E. faecium* SROD by phylogenetic analyses of 16S rRNA gene sequences. The fumarate reductase activities of the SROD5 and SROD strains were 42.13 and 37.05 mM NADH oxidized/min/mg of cellular nitrogen (N), respectively. Supplementation of rumen fermentation in vitro with the SROD5 and SROD strains produced significantly higher propionate, butyrate, and total volatile fatty acid (VFA) concentrations than controls at 12 h; VFA concentrations tended to increase after 24 h of incubation. The generated CH_4 concentration was significantly lower in the SROD5 and SROD treatment groups after 24 h of incubation. These findings indicate that *E. faecium* SROD has potential as a direct-fed microbial additive for increasing total VFAs while decreasing CH_4 production in rumen fermentation in vitro.

Keywords: *Enterococcus faecalis* SROD5, *Enterococcus faecium* SROD, fumarate reductase activity, methane, rumen fermentation in vitro

Introduction

CH_4 is a major end product of anaerobic fermentation in the rumen, accounting for approximately 95% of global animal enteric CH_4 [12]. CH_4 is a greenhouse gas, and reduction of CH_4 emissions is desirable. Increasing the number and activity of non-methanogenic H_2 utilizers is a promising method to reduce CH_4 emissions [24]. Sirohi *et al.* [34] reported that this could be achieved by diverting H_2 from CH_4 production to alternative electron-sink metabolic pathways. One such pathway involves the biochemical reduction of fumarate. Fumarate is an intermediate compound in the succinate–propionate pathway, which occurs in the rumen; fumarate is reduced to succinate by fumarate reductase [21]. Fumarate reductase catalyzes the reductive reaction, and fumarate functions as an electron acceptor during anaerobic growth [35].

Fumarate reduction plays an important role in the maintenance of a low partial H_2 pressure in the rumen [1]. The relatively high redox potential of fumarate/succinate coupling allows oxidation of various metabolic H_2 donors, including nicotinamide adenine dinucleotide (NADH), lactate, and formate, by fumarate [11]. Lamb rumens typically contain 10^7 cells/ml of fumarate-reducing bacteria [6]. One of these fumarate-reducing bacteria is *Mitsuokella jalaludinii* [23]; however, it is anaerobic, and is therefore difficult to cultivate and use in probiotics. *Enterococcus faecalis* has also been reported to have the ability to convert fumarate to succinate [13, 29-31]. *E. faecalis* is a facultative anaerobe, and can therefore be cultivated aerobically and also survive under the anaerobic conditions in the rumen. However, *E. faecalis* is not included in the list of probiotics that are acceptable for use in South Korea (Ministry of Agriculture, Food and Rural Affairs, 2015).

Enterococcus faecium is closely related to *E. faecalis* [33], and it has long been used as a probiotic. *E. faecium* is a component of the rumen microbiome that commonly colonizes the intestinal tract of host animals [15]. Ribeiro *et al*. [28] reported that *E. faecium* also increased the levels of organic acids such as acetate, propionate, and succinate. Thus, it is widely applied as a dietary supplement for chickens, sows, piglets, finishing pigs, calves, and fattening cattle [3]. Unlike *E. faecalis*, *E. faecium* is included in the list of probiotics that are acceptable for use in South Korea (Ministry of Agriculture, Food and Rural Affairs, 2015). Since *E. faecium* is closely related to *E. faecalis*, the likelihood that it can reduce fumarate to succinate is high. Therefore, this study was conducted to isolate and identify *Enterococcus* strains and to determine their fumarate reductase activities. Furthermore, the effects of supplementation with these microbes on rumen fermentation in vitro were investigated. The results of the present study might help to improve ruminal fermentation and to reduce CH_4 production by ruminants.

Materials and Methods

Animals and Sampling

Korean native goats (KNGs, *Capra hircus coreanae*) reared in Hwasun, South Korea were used as the experimental animals. KNGs of 45 ± 2 kg were fed Timothy hay and commercial concentrate (Woosung Co., Korea) at 2% body weight twice per day. The chemical composition (dry matter) of the commercial concentrate and Timothy hay used in this experiment were 18.62% and 15.89% crude protein, 2.93% and 3.87% ether extract, 7.23% and 26.80% crude fiber, 9.32% and 7.83% ash, and 76.44% and 56.95% total digestible nutrients, respectively. Rumen fluids containing timothy hay were collected from the rumens of slaughtered KNGs. The samples were strained through four layers of surgical gauze, and then immediately placed in amber bottles with oxygen-free headspace. The bottles containing the rumen fluid were sealed, maintained at 39°C, and immediately transported to the laboratory for isolation of enterococci.

Isolation of Fumarate-Reducing Enterococci

Enterococcosel agar (Becton, Dickinson, East Rutherford, NJ, USA) was used for isolation of enterococci. The medium was prepared and autoclaved for 15 min at 121°C, after which the rumen fluid was serially diluted from 10^{-5} to 10^{-7}, plated, and incubated in an anaerobic jar for 24 to 48 h at 39°C.

PCR Amplification, Amplified Ribosomal DNA Restriction Analysis, DNA Sequencing, and Phylogenetic Analysis

The 27f (AGAGTTTGATCMTGGCTCAG) and 1492r (TACGGY TACCTTGTTACGACTT) primers [16] were used for amplification of the gene encoding 16S ribosomal RNA (16S rDNA). Similarities and differences among isolates were determined by amplified ribosomal DNA restriction analysis (ARDRA). Polymerase chain reaction (PCR) products were double digested with the HaeIII and HhaI restriction endonucleases (TaKaRa, Japan) at 37°C for 5 h. The digested DNA samples were then separated by electrophoresis at 170 V for 80 min using MetaPhor agarose gel (Lonza, NJ, USA), and then they were visualized using a Kodak Gel Logic 200 imaging system (Eastman Kodak Company, NY, USA). The isolates with different band patterns obtained from ARDRA were subsequently purified using a QIAquick PCR Purification Kit (Qiagen, Valencia, CA, USA). Purified 16S rDNA PCR products were sent to Macrogen Korea for DNA sequencing. The sequenced fragments were assembled using the SeqMan software (DNA Star, Lasergene Software, Madison, WI, USA). The gene sequences were then compared with 16S RNA gene sequences available in GenBank using the Basic Local Alignment Search Tool (BLAST) algorithm (http://www.ncbi.nlm.nih.gov/BLAST/) and the EzTaxon server (http://ezbiocloud.net). Approximate phylogenetic affiliations were determined by aligning the gene sequences with those of closely related species using ClustalW ver. 1.6. A phylogenetic tree was constructed using the neighbor-joining (NJ) method with pair-wise gap removal, and distance matrices were calculated according to the method described by Kimura [14]. Finally, the two-parameter NJ method in the PHYLIP package was employed and bootstrap analysis was performed by resampling the data 1,000 times to evaluate the stability of the phylogenetic tree. Only bootstrap values greater than 50% are shown on the internal nodes.

Determination of Fumarate Reductase Activity

Preparation of the microbial membrane fraction. Of the 22 colonies isolated and identified, we selected two enterococci strains (*E. faecium* SROD and *E. faecalis* SROD5) for determination of their fumarate reductase activities. The microbes were cultivated using de Man, Rogosa, and Sharpe (MRS) broth. Cell growth was monitored based on the OD at 600 nm (approximately 1.50). Samples were divided into nine replicates and then separated into three groups for sonication (15, 20, and 25 min). Cells were harvested during the late log phase of growth by centrifugation (20,000 ×*g*, 10 min). The pellets were then resuspended in 5 ml of 50 mM KPi buffer, after which the cells were disrupted by ultrasonication (Ultrasonic Homogenizer 4710 Series; Cole-Parmer Instrument Corp., IL, USA) until 95% of the cells were disrupted [23]. To accomplish this, a titanium needle probe was immersed in the samples to a depth of approximately 5 mm. Samples were kept in a salt ice bath during cell disruption to prevent overheating (30 sec sonication and 30 sec rest per duty cycle; each duty cycle was 0.5 sec with generator acting for 0.5 sec intervals with 0.5 sec of rest). Unbroken cells were Gram stained and counted under a microscope (Olympus CX31, Japan). The unbroken cells were then removed by centrifugation (20,000 ×*g*, 10 min) [1].

Protein content determination. The protein content of the samples

was determined using a Quick Start Bradford Protein Assay with bovine serum albumin as a standard protein. The protein content of the control (untreated medium) was subtracted from the protein content of the samples (medium + microorganism). The volume of enzymes in the sample was then determined as follows:

$$M_iV_i = M_fV_f$$

where

M_i = sample protein content (μg/ml), as determined by Bradford assay

V_i = x (initial volume needed)

M_f = final protein content needed for the assay

V_f = enzyme assay volume (final volume needed)

Assay for enzyme activity. The fumarate reductase activity was assayed by determining the rate of NADH oxidation. The standard assay mixture (1.5 ml) contained 5 mM fumarate, 0.15 mM NADH, and an enzyme sample (1 mg protein/ml assay mixture) in 50 mM KPi buffer. The reaction was initiated by adding an enzyme sample at room temperature, after which the NADH content was determined by measuring the absorbance at 340 nm. The activity of NADH oxidase was estimated from the initial rate of NADH oxidation in the absence of fumarate, and this value was subtracted from the initial rate of NADH oxidation in the presence of fumarate to obtain the fumarate reductase activity, which was expressed as mM NADH oxidized/min/cellular N. This value was used to represent enzyme quantity per cell mass because approximately 95% of the cells had been disrupted by ultrasonication [1].

Cultivation of Microorganisms

Briefly, MRS broth was prepared according to the manufacturer's protocol, autoclaved, and cooled. Frozen stock cultures of *E. faecium* SROD and *E. faecalis* SROD5 were thawed and inoculated (1%) into bottles containing the cooled medium. Samples were then incubated in a horizontal shaking incubator (120 rpm) (Hanbaek Scientific Co., Korea) at 39°C until the optical density reached approximately 1.5, which was equivalent to 7.0×10^8 CFU/ml and 7.2×10^8 CFU/ml for *E. faecium* SROD and *E. faecalis* SROD5, respectively. The cultures were subcultured three times on the same medium to ensure full activity of the microorganisms.

Rumen Fermentation In Vitro

Ruminal contents were obtained from 48-month-old rumen-cannulated Holstein Friesian cattle with body weights of 600 ± 47 kg that had been fed twice a day with concentrate feed (NongHyup Co., Korea) and rice straw at a 2:8 ratio. Collection was performed by hand, after which the contents were squeezed and strained through four layers of cheese cloth. Ruminal fluid was pooled in prewarmed amber bottles, sealed, and immediately transported to the laboratory; the temperature was maintained at 39°C. Buffer solution was prepared according to the procedure described by Asanuma *et al*. [2] with the following composition in mg/l: dipotassium phosphate (K_2HPO_4), 450; monopotassium phosphate (KH_2PO_4), 450; magnesium sulfate heptahydrate ($MgSO_4 \cdot 7H_2O$), 190; calcium chloride dehydrate ($CaCl_2 \cdot 2H_2O$), 120; sodium chloride (NaCl), 900; cysteine hydrochloride ($C_3H_7NO_2S \cdot HCl$), 600; ammonium sulfate ($(NH_4)_2SO_4$), 900; trypticase peptone (BBL; Becton Dickinson), 1,000; and yeast extract (Difco Laboratories, MI, USA), 1,000. The prepared medium was then autoclaved at 121°C for 15 min and maintained in a 39°C water bath and flushed with N_2. The pH of the buffer was adjusted to 6.9 using 10 N NaOH, after which the particle-free rumen fluid was added (1:3 rumen fluid:buffer ratio). Next, 100 ml of buffered rumen fluid was anaerobically transferred to each of the serum bottles containing the 1% DM of maize silage substrate. The bottles were subsequently sealed with rubber septum stoppers and aluminum caps and incubated at 39°C for 12 or 24 h with shaking (100 rpm) [9]. The *E. faecium* SROD or *E. faecalis* SROD5 (1% of each) was then anaerobically inoculated into serum bottles and incubated in a HB-201SF shaking incubator (Hanbaek Scientific Co., Korea) at 39°C with horizontal shaking at 100 rpm [9]. The in vitro fermentation parameters were monitored for 12 and 24 h in triplicates. The positive control used was 30 mM disodium fumarate.

Analyses of Rumen Fermentation In Vitro Parameters

Fermentation parameters were monitored at the end of each incubation period. The pH was measured with a Pinnacle series M530p meter (Schott Instruments, Germany) immediately after uncapping each bottle. To measure the total gas, a press and sensor machine was used (Laurel Electronics, Inc., CA, USA). Briefly, a needle channel connected to the machine was extended into the sealed fermentation bottle to measure the positive pressure created by the gas built up in the headspace of the bottle at room temperature. A gas flow regulator was subsequently opened, allowing the gas to flow inside a syringe barrel. The plunger was pulled gradually until the pressure reading in the machine display reached zero and the volume of gas trapped inside the barrel was recorded as the total gas produced in milliliters.

Gas samples were also collected from the fermentation bottles into vacuum tubes and stored in a refrigerator until analysis by gas chromatography. One milliliter of the total gas produced was used to determine the amount of CH_4 emitted during the incubation period. Gas chromatography (HP 5890; Agilent Technologies, Germany) was performed using a TCD detector with a Carboxen 1006PLOT 30 m × 0.53 mm capillary column (Supelco, PA, USA). The quantity of CH_4 produced was estimated using the formula described by Ørskov and McDonald [26]. Peaks were identified by comparison with gas standards of known composition. Standards with $R^2 = 0.999$ were prepared prior to sample analysis. The CH_4 concentrations were calculated in parts per million (ppm).

Additionally, 1 ml of fermenta from each of the serum bottles was immediately centrifuged at 16,609 ×*g* for 10 min at 4°C using a Micro 17TR centrifuge (Hanil Science Industrial Co. Ltd., Korea). The supernatant was kept in 1.5 ml Eppendorf tubes and frozen at –80°C until VFA analysis. The samples in Eppendorf tubes were

thawed at room temperature, after which they were filtered through 0.2 μm Millipore filters for determination of VFA concentrations. Standards with R^2 = 0.999 were prepared prior to sample analysis. VFA concentrations were measured using high-performance liquid chromatography (Agilent Technologies 1200 series) with a UV detector set at 210 and 220 nm. A MetaCarb 87H (Varian, Germany) column was used in the determination of fermentation products with application of 0.0085 N H_2SO_4 buffer at a rate of 0.6 ml/min [36]. The VFA concentration in mM was calculated in ppm divided by the molecular weight.

Statistical Methods

Data were analyzed by analysis of variance using the general linear model for a randomized complete block design. All treatments were conducted in triplicates and Duncan's multiple range test was used to identify differences between specific treatments. A *p*-value <0.05 was considered to indicate statistical significance. All analyses were carried out using Statistical Analysis Systems ver. 9.1 [32].

Results and Discussion

Isolation of Fumarate-Reducing Enterococci and Determination of Fumarate Reductase Activity

The rumen is known to harbor many different strains of microorganisms. Enterococci are regularly isolated from the rumen [18], at approximately 4.81 ± 0.33 cells/ml to 6.02 ± 0.08 cells/ml of rumen content [19]. In the present study, 22 colonies of enterococci were isolated from rumen fluid samples of slaughtered KNGs and identified. Two of these, *E. faecium* SROD and *E. faecalis* SROD5, were found to be 99% and 100% similar to *E. faecium* ATCC 19434 and *E. faecalis* JCM 5803T, respectively, by phylogenetic analyses

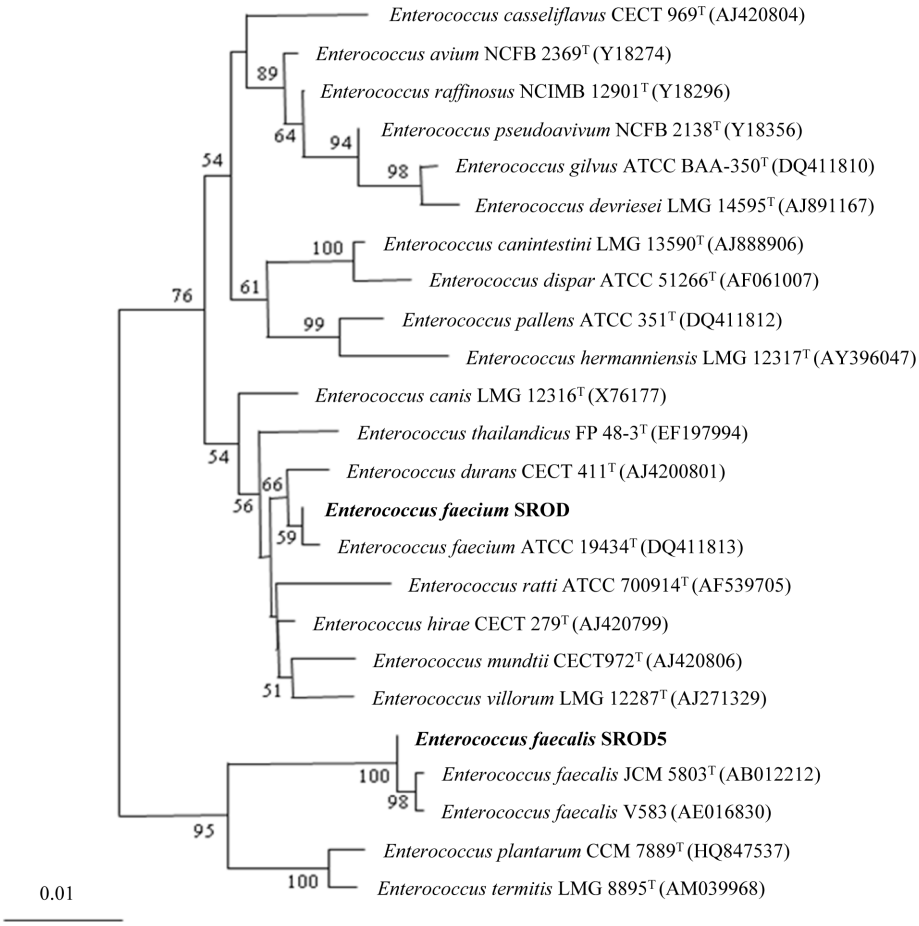

Fig. 1. Phylogenetic tree based on comparison of 16S rRNA gene sequences, indicating the taxonomic position of the *Enterococcus faecalis* SROD5 and *Enterococcus faecium* SROD isolates.

The phylogenetic tree was constructed using the neighbor-joining method. Bootstrap values, expressed as percentages of 1,000 replicates, are given at branching points. Only bootstrap values greater than 50% are shown on the internal nodes. The bar represents 0.01 substitutions per nucleotide position.

based on 16S rRNA gene sequences (Fig. 1). These two strains are the two microorganisms that are most frequently isolated from the rumen [17].

E. faecalis can convert fumarate to succinate [29] via a biochemical pathway that produces propionate. However, although *E. faecium* is approved for use as a probiotic in South Korea, *E. faecalis* is not (Ministry of Agriculture, Food and Rural Affairs, 2015). Since these two strains are closely related [33], it is likely that they both have the ability to convert fumarate to succinate. Hence, we investigated their fumarate reductase activities in the present study. Fig. 2 shows the fumarate reductase activity per cell mass of *E. faecium* SROD and *E. faecalis* SROD5 after sonication for 15, 20, or 25 min. Lower fumarate reductase activities were observed at 15 and 25 min of sonication, with the highest ($p < 0.05$) occurring at 20 min. After 20 min of sonication, *E. faecalis* SROD5 oxidized 42.13 mM NADH/min/mg of cellular N whereas *E. faecium* SROD oxidized 37.05 mM NADH /min/mg of cellular N.

In a study of *Misuokella jalaludinii* and *Veillonella parvula*, Mamuad *et al.* [23] determined enzyme activity after 15 min of sonication. In the present study, the enzyme activities of *E. faecalis* SROD5 and *E. faecium* SROD were determined after 20 min of sonication. We observed higher enzyme activities in *E. faecalis* SROD5 and *E. faecium* SROD than those in *M. jalaludinii* and *V. parvula.* These results indicated that the sonication time affects the enzyme activity of microorganisms. Moreover, the fumarate reductase activity of *E. faecalis* SROD5 tended to be higher than that of *E. faecium* SROD. The findings on the fumarate reductase activity of *E. faecium* SROD indicate that it has the ability to convert fumarate to succinate and is therefore a fumarate-reducing bacterium. Fumarate-reducing bacteria are hydrogenotrophic microorganisms that are also methanogen competitors. Competition with archaeal methanogenesis in the rumen for H_2 utilization could be an alternative means of reducing CH_4 production in ruminants. Thus, we further investigated the effects of *E. faecalis* SROD5 and *E. faecium* SROD on rumen fermentation in vitro.

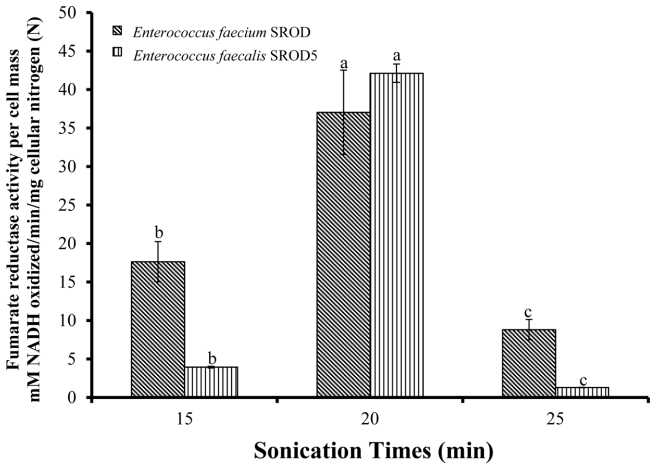

Fig. 2. Fumarate reductase activity per cell mass of *Enterococcus faecalis* SROD5 and *Enterococcus faecium* SROD at different sonication times.

The NADH oxidase activity was estimated from the initial rate of NADH oxidation in the absence of fumarate. This value was subtracted from the initial rate of NADH oxidation in the presence of fumarate to obtain the fumarate reductase activity, which is expressed as mM NADH oxidized per min per cellular nitrogen (N) in suspensions of disrupted cells. Values show the mean of three replicates, and bars indicate the standard error. Different letters (a > b > c) for each microbe (*Enterococcus faecalis* SROD5 or *Enterococcus faecium* SROD) indicate significant differences within the same time period (15, 20, and 25 min) as determined by Duncan's multiple range test ($p < 0.05$).

Effects of Supplementation with Fumarate-Reducing Enterococci on Rumen Fermentation In Vitro

After determining that *E. faecalis* SROD5 and *E. faecium* SROD show fumarate reductase activity, we investigated the effects of these microbes in the rumen. To accomplish this, we conducted rumen fermentation in vitro with supplementation of *E. faecalis* SROD5, *E. faecium* SROD, or disodium fumarate as a positive control. After incubation for 12 to 24 h, the pH decreased in all treatments except for the SROD5 treatment, which showed an increase from pH 5.43 to pH 5.58; however, none of the differences were significant (Fig. 3). Ruminal fermentation is inversely proportional to pH [22]; as ruminal fermentation increases, the pH decreases. Thus, the decrease in pH observed in all treatments except SROD5 indicates that ruminal fermentation was proceeding.

The total gas production during rumen fermentation in vitro supplemented with *E. faecalis* SROD5, *E. faecium* SROD, or disodium fumarate for 12 or 24 h of incubation is shown in Fig. 4. Gas production was the highest ($p < 0.05$) in the disodium fumarate treatment, and 42.67 and 78.33 ml of gas were observed at 12 and 24 h, respectively. Lopez *et al.* [21] also observed higher gas production in the disodium fumarate treatment group, similar to our results. The total volume of gas produced in the SROD5 group tended to be higher than in the control after 24 h of incubation.The increase in total gas production from 12 to 24 h of incubation was due to an increase in microbial fermentation; CO_2 is another end product of fermentation of fumarate to propionate via the succinate–propionate

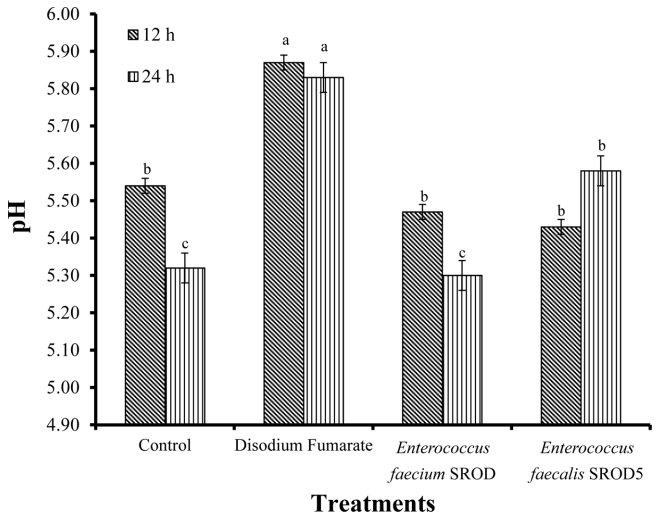

Fig. 3. pH of rumen fermentation in vitro supplemented with *Enterococcus faecalis* SROD5, *Enterococcus faecium* SROD, or disodium fumarate after 12 or 24 h of incubation.

Values show the mean of three replicates and bars indicate the standard error. Means for the same time period (12 or 24 h) marked with the same letters (a > b > c) did not differ significantly within treatments ($p < 0.05$), as determined by Duncan's multiple range test.

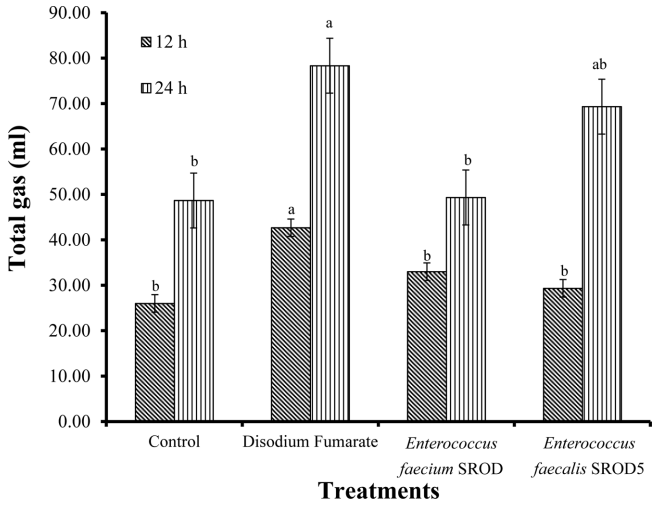

Fig. 4. Total gas produced during rumen fermentation in vitro supplemented with *Enterococcus faecalis* SROD5, *Enterococcus faecium* SROD, or disodium fumarate following 12 or 24 h of incubation.

Values show the mean of three replicates and bars indicate the standard error. Means for the same time period (12 or 24 h) marked with the same letters (a > b) did not differ significantly within treatments ($p < 0.05$), as determined by Duncan's multiple range test.

pathway [7].

The CH_4 concentration was found to be lowest ($p < 0.05$)

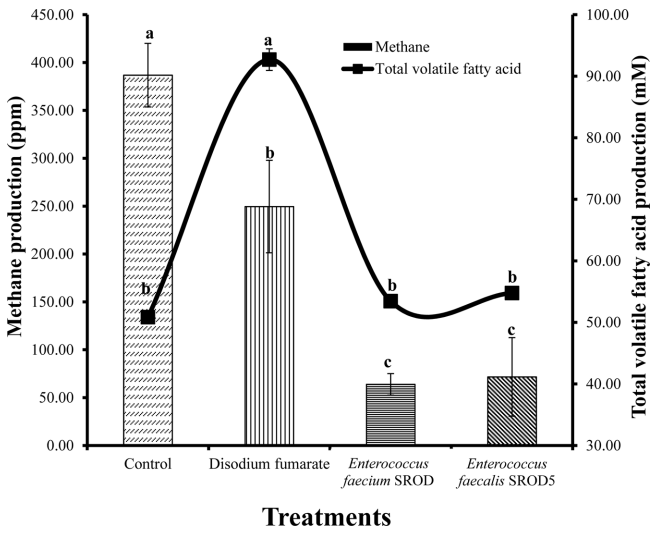

Fig. 5. Methane and total volatile fatty acid concentrations of rumen fermentation in vitro supplemented with *Enterococcus faecalis* SROD5, *Enterococcus faecium* SROD, or disodium fumarate after 24 h of incubation.

Total VFA is the sum of acetate, propionate, and butyrate. The VFA concentration is indicated in millimoles (mM) whereas CH_4 is indicated in parts per million (ppm). Values show the mean of three replicates and bars indicate the standard error. The bar graph indicates methane production and the line graph indicates the total volatile fatty acid production. Means on the same graph marked with the same letters (a > b > c) did not differ significantly within treatments ($p < 0.05$), as determined by Duncan's multiple range test.

in the *E. faecium* SROD (63.93 ppm) and *E. faecalis* SROD5 (71.69 ppm) treatments, followed by the disodium fumarate (249.60 ppm) and control treatments (386.88 ppm) (Fig. 5). Significantly lower CH_4 concentrations were observed in response to SROD and SROD5 supplementation, similar to the results observed following supplementation with *M. jalaludunii* [22] and *E. faecium* [27]. Moreover, the decrease in CH_4 production observed following the addition of disodium fumarate in this study is concordant with the decrease observed following addition of sodium fumarate by Lopez *et al.* [21]. H_2 is one of the key elements associated with CH_4 reduction because it is a major substrate for CH_4 production [22]. H_2 was not detected in this study (data not shown), which could indicate that it was consumed during the production of VFAs [20] such as fumarate [5, 22] or that it was used as a substrate for CH_4 production [5]. During conversion of fumarate to succinate, molecular H_2 serves as an electron donor for reduction of fumarate to succinate [8] and hence competes with methanogens during utilization of H_2. *E. faecium* SROD might have competed with methanogens

for H_2 consumption, which explains the lower CH_4 concentrations observed in this study.

Concentrations of microbial metabolites are often used as indicators of microbial activity [25]. VFAs are the end products of rumen fermentation. These compounds represent the main supply of metabolizable energy for ruminants [38], accounting for up to 80% of their maintenance energy [4]. The quantity of VFA produced depends on the quantity and composition of substrate [37] as well as the types of microorganisms present [22], and the metabolic pathway involved [23]. Acetate, butyrate, and propionate are the main VFAs in the rumen after microbial fermentation. In this study, the disodium fumarate treatment had the highest ($p < 0.05$) acetic acid concentration after 24 h of incubation, whereas the *E. faecalis* SROD5 and *E. faecium* SROD treatments tended to have higher acetic acid concentrations than the control after 12 and 24 h of incubation (Table 1). Demeyer and Henderickx [7] reported that fumarate can be converted into propionate and acetate via different pathways. This corroborates the increased acetate concentration observed in the disodium fumarate treatment in this study, which is also similar to the acetate production results obtained by Carro and Ranilla [5], with addition of disodium fumarate.

Propionate is the major substrate of hepatic glucogenesis for production of glucose, which is required for synthesis of milk lactose [10]. The propionic acid concentrations of the *E. faecium* SROD (10.24 mM) and *E. faecalis* SROD5 (10.47 mM) treatments were significantly higher than those of the control at 12 h and tended to be higher after 24 h of incubation. The significant increases in propionic acid concentrations observed in this study (12 h) were comparable to results reported by Pang *et al*. [27] and Carro and Ranilla [5], following supplementation with *E. faecium* and disodium fumarate, respectively. Although the differences were not significant, the increase in propionic acid observed after 24 h of incubation with *E. faecium* SROD or *E. faecalis* SROD5 was similar to the results observed following supplementation with *M. jalaludinii* and *V. parvula* [22]. The increase in propionate might be due to the conversion of fumarate to succinate, which leads to propionate production.

Furthermore, the butyric acid concentration was higher ($p < 0.05$) in the SROD5 and SROD treatments than in controls, at 6.25 and 6.23 mM after 12 h of incubation. After 24 h of incubation, the butyric acid concentration tended to be the highest in the *E. faecalis* SROD5 treatment, followed by the *E. faecium* SROD, disodium fumarate, and control treatments, respectively, although the differences were not significant. The butyric acid concentration tended to increase after 24 h of incubation, similar to results reported by Mamuad *et al*. [22]. However, the opposite was observed by Pang *et al*. [27], who found that butyric acid decreased significantly with supplementation of *E. faecium*. Furthermore, significantly higher total VFAs were observed at 12 h in the SROD5 and SROD treatments, at 49.85 mM and 49.07 mM, respectively. Conversely, the total VFA concentrations in the *E. faecalis* SROD5 and *E. faecium* SROD treatments tended to be higher than in the control.

E. faecium SROD isolated from KNGs produces fumarate reductase. Overall, the results of this study indicate that

Table 1. Volatile fatty acid concentration of rumen fermentation in vitro supplemented with *Enterococcus faecalis* SROD5, *Enterococcus faecium* SROD, or disodium fumarate.

Time (h)	Parameters (mM)	Treatments				SEM
		Control	Disodium fumarate	*Enterococcus faecium* SROD	*Enterococcus faecalis* SROD5	
12	Acetate	31.22 ± 0.293^{a}	19.11 ± 2.529^{b}	32.61 ± 0.338^{a}	33.13 ± 0.033^{a}	0.460
	Propionate	8.33 ± 0.085^{c}	12.90 ± 0.389^{a}	10.24 ± 0.616^{b}	10.47 ± 0.158^{b}	0.180
	Butyrate	5.74 ± 0.605^{b}	5.44 ± 0.192^{b}	6.23 ± 0.273^{a}	6.25 ± 0.139^{a}	0.180
	Total VFA	45.28 ± 0.811^{b}	37.44 ± 1.962^{c}	49.07 ± 0.781^{a}	49.85 ± 0.261^{a}	0.550
24	Acetate	33.71 ± 3.136^{b}	57.34 ± 6.205^{a}	34.36 ± 0.997^{b}	35.71 ± 1.576^{b}	1.770
	Propionate	9.95 ± 0.595^{b}	27.87 ± 1.156^{a}	11.40 ± 1.344^{b}	10.39 ± 0.854^{b}	0.600
	Butyrate	7.22 ± 0.256	7.49 ± 0.726	7.70 ± 0.992	8.37 ± 0.634	0.380
	Total VFA	50.87 ± 2.494^{b}	92.71 ± 8.059^{a}	53.46 ± 0.637^{b}	54.78 ± 1.138^{b}	1.810

Values are shown as the mean ± standard deviation of three replicates after 12 or 24 h of incubation. Means in the same row marked with different superscript letters (a > b > c) differed significantly within treatments ($p < 0.05$), as determined by Duncan's multiple range test. Total VFA is the sum of acetate, propionate, and butyrate. SEM = standard error of the mean; h = hour.

E. faecium SROD has potential as a direct-fed microbial additive to increase total VFAs while decreasing the CH_4 concentration during rumen fermentation in vitro. This study tested in vitro fermentation only; further quantification of the metabolic products of *E. faecium* SROD will be required, along with further testing in different animal species.

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