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Effects of *Cordyceps militaris* on the growth of rumen microorganisms and in vitro rumen fermentation with respect to methane emissions

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ABSTRACT

This experiment was designed to investigate the effects of different concentrations (0.00, 0.10, 0.15, 0.20, 0.25, and 0.30 g/L) of dried *Cordyceps militaris* mushroom on in vitro anaerobic ruminal microbe fermentation and methane production using soluble starch as a substrate. Ruminal fluids were collected from Korean native cattle, mixed with phosphate buffer (1:2), and incubated anaerobically at 38°C for 3, 6, 9, 12, 24, 36, 48, and 72 h. The addition of *C. militaris* significantly increased total volatile fatty acid and total gas production. The molar proportion of acetate was decreased and that of propionate was increased, with a corresponding decrease in the acetate:propionate ratio. As the concentration of *C. militaris* increased from 0.10 to 0.30 g/L, methane and hydrogen production decreased. The decrease in methane accumulation relative to the control was 14.1, 22.0, 24.9, 39.7, and 40.9% for the 0.10, 0.15, 0.20, 0.25, and 0.30 g/L treatments, respectively. Ammonia-N concentration and numbers of live protozoa decreased linearly with increasing concentrations of *C. militaris.* The pH of the medium significantly decreased at the highest level of *C. militaris* compared with the control. In conclusion, *C. militaris* stimulated mixed ruminal microorganism fermentation and inhibited methane production in vitro. Therefore, *C. militaris* could be developed as a novel compound for antimethanogenesis.
Key words: *Cordyceps militaris*, methane, protozoa, rumen fermentation

INTRODUCTION

Methane (CH_4) produced as a result of digestible structural carbohydrate fermentation in the rumen represents 7 to 10% feed energy loss to the host animal (Takahashi et al., 1997; Takahashi, 2001) and has received attention as a potential contributor to global warming (Leng, 1991; Moss, 1993). Because methane production is negatively correlated with energy utilization in ruminants (Ørskov et al., 1968), many compounds have been tested in vitro and in vivo as methane inhibitors (Czerkawski and Breckenridge, 1972; Martin and Macy, 1985). Methanogenic archaea were metabolically correlated with ciliate protozoa (Stumm et al., 1982; Newbold et al., 1995), and elimination of ciliate protozoa from the rumen reduced methane emissions by 30 to 45% (Jouany et al., 1981; Itabashi et al., 1984; Ushida et al., 1986). Several compounds have the potential to reduce methane production from ruminants although their long-term effects have not been well established. Some compounds are toxic or may not be economically feasible (Hristov et al., 2013), or an adaptive response may occur in some bioactive compounds after supplementation (Wallace et al., 2002).

Cordyceps militaris, a traditional Chinese medicinal mushroom, is an entomogenous fungus belonging to the Ascomycotina. The mushroom is traditionally called "DongChung HaCho" in Korea meaning "summer-plant and winter-worm." During the past several decades, many kinds of bioactive constituents from *Cordyceps* spp. have been isolated and characterized. These include cordycepic acid (D-mannitol), cordycepin, ophicordin, polysaccharides, amino acids, galactosaminoglycan, nucleic acids, steroids, and L-tryptophan (Tang and Eisenbrand, 1992; Namba, 1993; Huang et al., 2003). In addition, *C. militaris* showed several therapeutic effects, including immunoregulative (Zhu et al., 1998a,b; Ahn et al., 2000; Zhou et al., 2002), anticancer (Müller et al., 1977), antibacterial (Ahn et al., 2000), antifungal (Sugar and McCaffrey, 1998), larvicidal (Kim et al., 2002), and antioxidant (Li et al., 2001; Tsai et al., 2001) effects. *Cordyceps militaris* mycelia have been shown to alter in vitro rumen microbial fermentation with increased production of gas and VFA, cellulose digestion, and cellulolytic enzyme activities (Yeo et al., 2009, 2011). But no information exists with respect

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to *C. militaris* modulating methane production in the rumen. Therefore, the present study was conducted to observe the effects of *C. militaris* on ruminal microorganism fermentation with particular reference to methane production in vitro.

MATERIALS AND METHODS

Sample Preparation

Because *Cordyceps* are very difficult to collect due to their very small size and restricted area of growth, mass production of these fungi has been established through artificial cultivation. Dried *C. militaris* was cultured on floral medium composed of gluten, soybean protein, beer yeast, and corn steep liquor (culturing method and medium composition were patented in Korea, patent registration No.1006442430000; Lee, 2006) obtained from EuGene Bio Farm (Hwaseong City, Gyeonggi Province, Korea). The manufacturer reported that *C. militaris* mycelia used in the present study contained about 2.3 times more cordycepin (1.6 mg/g of DM) than *C. militaris* traditionally cultured on faunal pupae (0.7 mg/g of DM). It contained 8.6% moisture, 76.2% CP, 12.2% crude fiber, 1.0% ether extract, 3.2% crude ash, and 7.4% nitrogen-free extract.

In Vitro Batch Fermentation

The anaerobic culture techniques of Hungate (1950) with modifications (Bryant and Burkey, 1953; Bryant, 1972) were carried out for all incubations and the experimental procedures were the same as those described in a previous study (Yeo et al., 2009) except that 200 mg of soluble potato starch (S2004; Sigma-Aldrich Korea, Yongin City, Gyeonggi-do, Korea) was used as a carbon source.

Dried *C. militaris* was added gravimetrically to achieve final concentrations of 0.00, 0.10, 0.15, 0.20, 0.25, and 0.30 g/L. The bottles (3 replicates per treatment) were closed with butyl rubber stoppers under the Hungate anaerobic gassing system hooked to a source of oxygen-free gas, sealed with aluminum caps, and placed in an incubator at 38°C for 3, 6, 9, 12, 24, 36, 48, and 72 h without shaking. The experimental design was a complete randomized design with 3 replications per treatment.

Total, Hydrogen, and Methane Gas Production

At the end of each incubation time, a needle attached to a glass syringe was inserted through the butyl rubber stopper, and the volume of gas exceeding 1 atm was measured through displacement of the syringe plunger using the technique of Fedorak and Hrwdey (1983) with modifications (Callaway and Martin, 1996). A 0.5-mL subsample of gas was analyzed for hydrogen and methane content by GC (model CP-3800, Varian Inc., Palo Alto, CA) using a molecular sieve 13×, 45- to 60-mesh column (2.0 mm × 3.2 mm × 2.0 mm, stainless steel) and a thermal conductivity detector (oven temperature = 60°C, injector and thermal conductivity detector temperature = 120°C, flame-ionization detector temperature = 200°C). The carrier gas (N_2) flow rate was 50 mL/min.

pH, NH_3-N, and VFA

After determination of gas production, the bottles were uncapped, and pH of the culture fluid was determined using a pH meter (MP 230, Mettler-Toledo, Greifensee, Switzerland). For analysis of ammonia-N and VFA, 1 mL of 25% meta-phosphoric acid was added to 5 mL of fermentation fluid and centrifuged (10,000 × *g* for 10 min at 4°C); supernatants were stored at −30°C until analysis. Volatile fatty acids were analyzed by GC (model GC-14B, Shimadzu Co. Ltd., Tokyo, Japan) using a Thermon-3000 5% Shincarbon A column (1.6m × 3.2 mm i.d., 60 to 80 mesh, Shinwakako, Kyoto, Japan) and flame-ionization detector (column temperature = 130°C, injector and detector temperature = 200°C). The carrier gas (N_2) flow rate was 50 mL/min. The micro-diffusion method was used to determine NH_3-N (Conway, 1962).

Microbial Populations

Total viable bacteria and fungi in the culture fluid were enumerated by the method of Holdeman et al. (1977) and Joblin (1981) using anaerobic roll tubes. Samples were fixed in methylgreen-formalin-saline (MFS) solution consisting of 900 mL of distilled water, 100 mL of 35% formaldehyde solution, 0.6 g of methylgreen, and 8.0 g of NaCl before enumeration of rumen protozoa by the method of Ogimoto and Imai (1981). Protozoa fixed in MFS were diluted in the same solution and counted under a microscope with a plankton-counting glass (cat. no. 900, Hausser Scientific, Blue Bell, PA).

Relative Quantification of Specific Ruminal Microbes

Total nucleic acid was extracted from the incubated rumen samples using the modified bead-beating protocol (Yu and Morrison, 2004) with the QIAamp DNA mini kit (Qiagen, Valencia, CA). This was ac-

complished by taking a 1.0-mL aliquot from the culture medium using a wide-bore pipette to ensure collection of a homogeneous sample. Nucleic acid concentrations were measured using a NanoDrop Spectrophotometer (Thermo Scientific, Wilmington, DE).

Quantitative (**q**)PCR assays for enumeration of methanogenic archaea, ciliate protozoa, and cellulolytic bacterial species (*Fibrobacter succinogenes*, *Ruminococcus flavefaciens*, *Ruminococcus albus*) were performed according to the methods described by Koike and Kobayashi (2001), Denman and McSweeney (2006), and Denman et al. (2007) on a real-time PCR machine (CFX96 Real-Time system, Bio-Rad, Hercules, CA) using the SYBR Green Supermix (QPK-201, Toyobo Co. Ltd., Tokyo, Japan). The PCR primer sets used are shown in Table 1. They included group-specific primers for total bacteria as reference genes and species-specific primers for *F. succinogenes*, *R. flavefaciens*, *R. albus*, methanogenic archaea, and ciliate protozoa. All microbial data were analyzed for calculating relative expressions to total bacteria (Denman and McSweeney, 2005). The values of cycle threshold (**Ct**) after real-time PCR were used to determine the fold change of different microbial populations relative to control (Yuan et al., 2006). Abundance of these microbes was expressed by the equation relative quantification $= 2^{-[\Delta Ct\ (Target) - \Delta Ct\ (Control)]}$, where Ct represents threshold cycle. All qPCR reaction mixtures (final volume of 25 μL) contained forward and reverse primers (10 pmol each), the iQ SYBR Green Supermix (Toyobo Co. Ltd.), and DNA template ranging from 10 to 100 ng. A negative control without template DNA was used in every qPCR assay for each primer. The PCR amplification of the target DNA was conducted following the references in Table 1.

Computation of Data and Statistical Analysis

To give a more precise estimate of gas production throughout fermentation, the following calculation was used to analyze the kinetic data, as described by Ørskov and McDonald (1979):

$$G_P = a + b(1 - \exp^{-c \times \text{time}}),$$

where G_P is gas production (mL/0.1 g DM of substrate) at time t; a, b, and c are the scaling factor for Y-axis intercept (mL/0.1 g of DM), potential gas production (mL/0.1 g of DM), and the rate constant for gas production per hour (h^{-1}), respectively. Gas production rate was fitted to the model by using the nonlinear (NLIN) procedure (SAS Institute, 2011) using Marquardt's algorithm while varying a, b, and c. Effective gas production (EG_P: substrate availability) from the culture was estimated as $EG_P = a + b[c_d/(c_d + c_p)]$, where c_d is a gas production rate constant, and c_p is a passage rate constant assumed to be 0.05/h (NRC, 1989).

Data obtained from the experiment were analyzed using the SAS/OR (SAS Institute, 2011) software package and differences were tested by Duncan's multiple range test. Significance was declared at $P < 0.05$.

RESULTS

Table 2 shows the effects of *C. militaris* on cumulative gas production and its parameters at different incubation times. Gas production was linearly increased by the addition of *C. militaris* at all incubation times. The potential gas production ($a + b$) was significantly higher for *C. militaris* treatments than for the control treatment. In all treatments, cumulative gas production by mixed rumen microorganisms rapidly increased from 3 to 12 h of incubation. The addition of *C. militaris* significantly increased ($P < 0.05$) total gas production compared with the control except at 6, 9, and 24 h of incubation for 0.10 and 0.15 g/L treatments. The highest total gas production was seen

Table 1. Primers (F = forward; R = reverse) for real-time PCR assay

Target species	Primer sequence (5′→3′)	Size (bp)	Reference
Total bacteria	F: CGG CAA CGA GCG CAA CCC R: CCA TTG TAG CAC GTG TGT AGC C	130	Denman and McSweeney (2006)
Ruminococcus albus	F: CCC TAA AAG CAG TCT TAG TTC G R: CCT CCT TGC GGT TAG AAC A	175	Koike and Kobayashi (2001)
Ruminococcus flavefaciens	F: CGA ACG GAG ATA ATT TGA GTT TAC TTA GG R: CGG TCT CTG TAT GTT ATG AGG TAT TAC C	132	Denman and McSweeney (2006)
Fibrobacter succinogenes	F: GTT CGG AAT TAC TGG GCG TAA A R: CGC CTG CCC CTG AAC TAT C	121	Denman and McSweeney (2006)
Methanogenic archaea	F: TTC GGT GGA TCD CAR AGR GC R: GBA RGT CGW AWC CGT AGA ATC C	140	Denman et al. (2007)
Ciliate protozoa	F: GAG CTA ATA CAT GCT AAG GC R: CCC TCA CTA CAA TCG AGA TTT AAG G	180	Skillman et al. (2006)

Table 2. Effects of different doses of *Cordyceps militaris* on in vitro cumulative gas production (at 3 to 72 h of incubation) by mixed rumen anaerobic microbial fermentation

	Dose (g/L)						Contrast		
Incubation time (h)	0.00	0.10	0.15	0.20	0.25	0.30	SEM	Linear	Quadratic	Cubic
Gas production (mL/0.1 g DM of substrate)										
3 h	4.57^{b}	8.77^{a}	8.60^{a}	10.37^{a}	9.13^{a}	9.73^{a}	0.55	0.002	0.017	0.200
6 h	5.57^{e}	9.20^{bc}	11.10^{bc}	12.50^{ab}	13.23^{ab}	15.80^{a}	1.00	0.001	0.550	0.480
9 h	10.77^{c}	14.43^{bc}	15.20^{b}	17.47^{ab}	20.50^{a}	21.27^{a}	0.98	<0.001	0.650	0.960
12 h	13.50^{d}	16.90^{c}	19.20^{c}	18.23^{c}	24.13^{b}	28.03^{a}	1.20	<0.001	0.052	0.043
24 h	17.03^{e}	19.13^{bc}	21.10^{b}	20.13^{b}	25.63^{a}	19.77^{b}	0.68	<0.001	0.002	0.014
36 h	17.00^{c}	21.13^{b}	22.47^{b}	23.17^{b}	26.97^{a}	21.17^{b}	0.77	<0.001	<0.001	0.058
48 h	18.27^{d}	21.50^{bc}	23.43^{b}	22.20^{bc}	28.50^{a}	20.67^{c}	0.80	<0.001	<0.001	0.005
72 h	19.40^{d}	21.60^{c}	23.57^{bc}	23.80^{b}	29.40^{a}	22.53^{bc}	0.77	<0.001	<0.001	<0.001
Gas production parameters[1]										
a (mL/0.1 g DM of substrate)	−1.38^{a}	3.12^{a}	1.81^{a}	5.06^{a}	−2.34^{a}	−3.62^{b}	0.25	0.056	0.052	0.280
b (mL/0.1 g DM of substrate)	20.05^{b}	18.57^{b}	21.53^{b}	18.14^{b}	30.49^{ab}	27.96^{a}	1.05	0.031	0.079	0.350
$a + b$ (mL/0.1 g DM of substrate)	18.68^{c}	21.69^{b}	23.34^{b}	23.20^{b}	28.16^{a}	22.35^{b}	0.55	<0.001	<0.001	0.004
k (G_P·h^{-1})	0.0983^{b}	0.0977^{b}	0.1123^{b}	0.1050^{b}	0.1453^{b}	0.2730^{a}	0.08	0.008	0.027	0.150
EG_P (%)	11.90^{d}	15.31^{c}	16.66^{bc}	17.25^{b}	20.27^{a}	18.46^{bc}	0.53	<0.001	<0.001	0.061

[a–d]Means (n = 3) with different superscripts in the same rows are different ($P < 0.05$).

[1]Gas production parameters, a, b, and c, for the negative exponential equation $G_P = a + b(1 - \exp^{-c \times time})$, where G_P is gas production (ml/0.1 g DM of substrate) of time t; a = gas production from the immediately soluble fraction; b = gas production from the insoluble fraction; c = the fractional rate of gas production per hour; $a + b$ = potential extent of gas production; EG_P = effective gas production rate from the cultures, calculated as $EG_P = a + b[k_d/(k_d + k_p)]$, where k_d (k) is a gas production rate constant, and k_p is a passage rate constant assumed to be 0.05 h^{-1}.

($P < 0.05$) in the 0.25 g/L treatment from 24 to 72 h of incubation.

Table 3 shows the effects of *C. militaris* on methane and hydrogen gas production. The addition of *C. militaris* reduced methane production linearly ($P < 0.05$) from 24 to 72 h, but a linear reduction of hydrogen gas production was seen only at 24 h of incubation. The largest reduction of methane production relative to the control was seen at 24 h of incubation, showing reductions of 14.1, 22.0, 24.9, 39.7, and 40.9% for 0.10, 0.15, 0.20, 0.25, and 0.30 g/L treatments, respectively.

A linear reduction of the concentration of ammonia-N by the addition of *C. militaris* was seen at 12 and 24 h of incubation (Table 4). Total VFA concentration was linearly increased ($P < 0.05$) by the addition of *C. militaris* from 24 to 72 h (Table 4), and corresponding decreases of pH were seen. At all levels of *C. militaris* addition at 24 h of incubation (Figure 1), the molar proportion of acetate was decreased ($P < 0.05$) compared with the control and that of propionate was increased ($P < 0.05$) in the 0.20 to 0.30 g/L treatments. This led to corresponding decreases in acetate:propionate ratio as the addition of *C. militaris* increased.

Figure 2 shows the effects of *C. militaris* on microbial populations in culture fluid after 24 h of incubation. The numbers of total and cellulolytic bacteria in the supernatant significantly increased ($P < 0.05$) at the highest dose level of *C. militaris* compared with the control. Significant decreases ($P < 0.05$) in the number of live protozoa and anaerobic fungi were seen in the 0.25 and 0.30 g/L treatments compared with the control, whereas numbers of dead protozoa remained similar between the treatments.

Real-time PCR analysis indicated that *C. militaris* significantly affected abundance of cellulolytic bacteria (*R. albus*, *R. flavefaciens*, and *F. succinogenes*), ciliate protozoa, and methanogenic archaea (Figure 3). The addition of *C. militaris* significantly decreased ($P < 0.05$) the abundance of *R. albus* in the 0.25 and 0.30 g/L treatments at 24 and 48 h of incubation, and for the 0.10, 0.20, and 0.30 g/L treatments at 12 h of incubation. Supplementation with *C. militaris* also decreased the abundance of *F. succinogenes* at 24 h except in the 0.15 and 0.20 g/L treatments but decreased responses were not shown at 12 and 48 h of incubation. On the other hand, *R. flavefaciens* in the 0.15, 0.25, and 0.30 g/L treatments was significantly increased ($P < 0.05$) at 24 h of incubation, and increased responses were shown for the 0.10 and 0.15 g/L treatments only at 12 and 48 h of incubation, respectively. A significant decrease ($P < 0.05$) in the abundance of ciliate protozoa was evident at 24 h of incubation when *C. militaris* was added at a level greater than 0.15 g/L. At 48 h of incubation, reductions in the abundance of ciliate protozoa

Table 3. Effects of different doses of *Cordyceps militaris* on methane (CH_4) and hydrogen (H_2) gas production (at 3 to 72 h of incubation) in supernatant of growing mixed rumen anaerobic microorganisms

	Dose (g/L)							Contrast		
Item	0.00	0.10	0.15	0.20	0.25	0.30	SEM	Linear	Quadratic	Cubic
Methane production (m*M*)										
3 h	1.37^{b}	1.95^{ab}	2.04^{ab}	2.55^{a}	2.02^{ab}	2.31^{a}	0.21	0.009	0.060	0.444
6 h	1.64	2.01	2.55	2.12	2.08	2.50	0.27	0.100	0.416	0.153
9 h	4.59	3.47	3.88	3.67	3.39	4.18	0.55	0.595	0.203	0.930
12 h	6.31	5.53	4.88	5.09	5.02	5.31	0.42	0.097	0.072	0.693
24 h	15.63^{a}	13.43^{b}	12.20^{bc}	11.73^{c}	9.43^{d}	9.23^{d}	0.45	<0.0001	0.189	0.730
36 h	23.33^{a}	19.22^{b}	17.52^{bc}	18.18^{bc}	15.35^{cd}	14.49^{d}	0.90	<0.0001	0.182	0.129
48 h	29.90^{a}	20.75^{d}	24.10^{bc}	26.83^{b}	22.00^{cd}	21.30^{cd}	0.95	0.001	0.292	<.0001
72 h	39.64^{a}	37.45^{ab}	35.13^{bc}	33.21^{c}	34.67^{bc}	32.35^{c}	1.25	0.001	0.232	0.590
Hydrogen production (m*M*)										
3 h	0.01^{d}	0.03^{bc}	0.02^{cd}	0.03^{a}	0.02^{cd}	0.03^{ab}	0.00	0.002	0.068	0.088
6 h	0.02^{b}	0.03^{ab}	0.03^{ab}	0.03^{ab}	0.02^{ab}	0.03^{a}	0.00	0.093	0.982	0.064
9 h	0.05	0.05	0.04	0.05	0.04	0.05	0.01	0.699	0.480	0.424
12 h	0.07^{ab}	0.07^{a}	0.06^{ab}	0.07^{ab}	0.05^{b}	0.07^{ab}	0.00	0.526	0.127	0.172
24 h	0.22^{a}	0.19^{b}	0.17^{b}	0.14^{c}	0.12^{c}	0.12^{c}	0.01	<0.0001	0.060	0.656
36 h	0.25 ^{ab}	0.25 ^{a}	0.20^{b}	0.24^{ab}	0.23^{ab}	0.22^{ab}	0.01	0.171	0.544	0.407
48 h	0.32^{ab}	0.27^{cd}	0.26^{cd}	0.35^{a}	0.24^{d}	0.29^{bc}	0.01	0.243	0.398	0.130
72 h	0.43^{bc}	0.50^{a}	0.40^{bc}	0.44^{b}	0.39^{c}	0.44^{b}	0.01	0.034	0.475	0.002

$^{a–d}$Means (n = 3) with different superscripts in the same rows are different ($P < 0.05$).

were seen only in the 0.25 and 0.30 g/L treatments. The effects of *C. militaris* addition on the abundance of methanogenic archaea were inconsistent. The 0.10 and 0.30 g/L treatments at 12 h and the 0.25 g/L treatment at 24 h of incubation decreased the abundance of methanogenic archaea; however, at 48 h of incubation,

Table 4. Effects of different doses of *Cordyceps militaris* on pH value, ammonia-N, and total VFA production (at 3 to 72 h of incubation) in supernatant of growing mixed rumen anaerobic microorganisms

	Dose (g/L)							Contrast		
Item	0.00	0.10	0.15	0.20	0.25	0.30	SEM	Linear	Quadratic	Cubic
pH										
3 h	6.47^{b}	6.54^{ab}	6.45^{ab}	6.46^{a}	6.46^{ab}	6.47^{a}	0.21	0.009	0.060	0.444
6 h	6.34	6.23	6.24	6.26	6.44	6.26	0.27	0.100	0.416	0.153
9 h	6.25	6.43	6.10	6.36	6.28	6.39	0.55	0.595	0.203	0.930
12 h	6.20	6.17	6.09	6.13	6.22	6.01	0.42	0.097	0.072	0.693
24 h	6.17^{a}	6.05^{b}	6.14^{bc}	6.02^{c}	5.95^{d}	5.78^{d}	0.45	<0.0001	0.189	0.730
36 h	6.00^{a}	5.95^{b}	5.89^{bc}	5.80^{bc}	5.65^{cd}	6.02^{d}	0.90	<0.0001	0.182	0.129
48 h	5.66^{a}	5.53^{d}	5.36^{bc}	5.37^{b}	5.21^{cd}	5.37^{cd}	0.95	0.001	0.292	<0.0001
72 h	5.48^{a}	5.45^{ab}	5.14^{bc}	5.16^{c}	5.14^{bc}	5.24^{c}	1.25	0.001	0.232	0.590
Ammonia-N (mg/dL)										
3 h	3.87	3.83	4.30	4.40	4.03	4.18	0.48	0.5842	0.5864	0.9696
6 h	4.43	4.03	4.27	4.80	4.40	4.26	0.36	0.8044	0.7165	0.2739
9 h	5.50	5.90	5.57	6.50	6.30	4.62	0.56	0.6417	0.0806	0.1742
12 h	5.83^{ab}	6.20^{a}	5.20^{ab}	5.43^{ab}	4.93^{b}	4.73^{b}	0.34	0.0077	0.7858	0.6087
24 h	6.47^{a}	5.90^{ab}	5.30^{bc}	4.93^{cd}	4.30^{de}	3.57^{e}	0.25	<0.0001	0.6838	0.5987
36 h	10.60	9.60	9.83	10.10	9.73	9.00	0.80	0.2921	0.8861	0.3672
48 h	17.37	15.33	16.50	16.80	20.87	16.40	2.90	0.6277	0.9833	0.272
72 h	19.60	18.50	21.30	20.07	22.33	22.53	1.88	0.1383	0.8039	0.7788
Total VFA production (*mM*)										
3 h	30.45	31.84	30.68	31.52	32.06	31.14	0.75	0.448	0.499	0.890
6 h	31.82	31.10	30.48	31.50	33.03	33.16	0.85	0.081	0.123	0.357
9 h	36.30	36.61	35.75	37.16	36.72	38.59	1.00	0.143	0.323	0.716
12 h	39.12	40.06	43.13	40.58	40.33	44.35	2.85	0.327	0.937	0.385
24 h	43.56^{c}	44.51^{c}	45.72^{bc}	47.92^{ab}	48.18^{ab}	48.89^{a}	0.80	<0.0001	0.507	0.478
36 h	46.98^{b}	54.55^{ab}	59.48^{a}	56.19^{ab}	65.28^{a}	65.52^{a}	3.71	0.002	0.566	0.548
48 h	49.93^{b}	59.76^{ab}	62.41^{a}	58.23^{ab}	67.56^{a}	66.16^{a}	3.60	0.006	0.390	0.388
72 h	59.39^{b}	61.64^{b}	59.88^{b}	69.55^{a}	66.97^{ab}	70.67^{a}	2.44	0.002	0.861	0.561

$^{a–e}$Means (n = 3) with different superscripts in the same rows are different ($P < 0.05$).

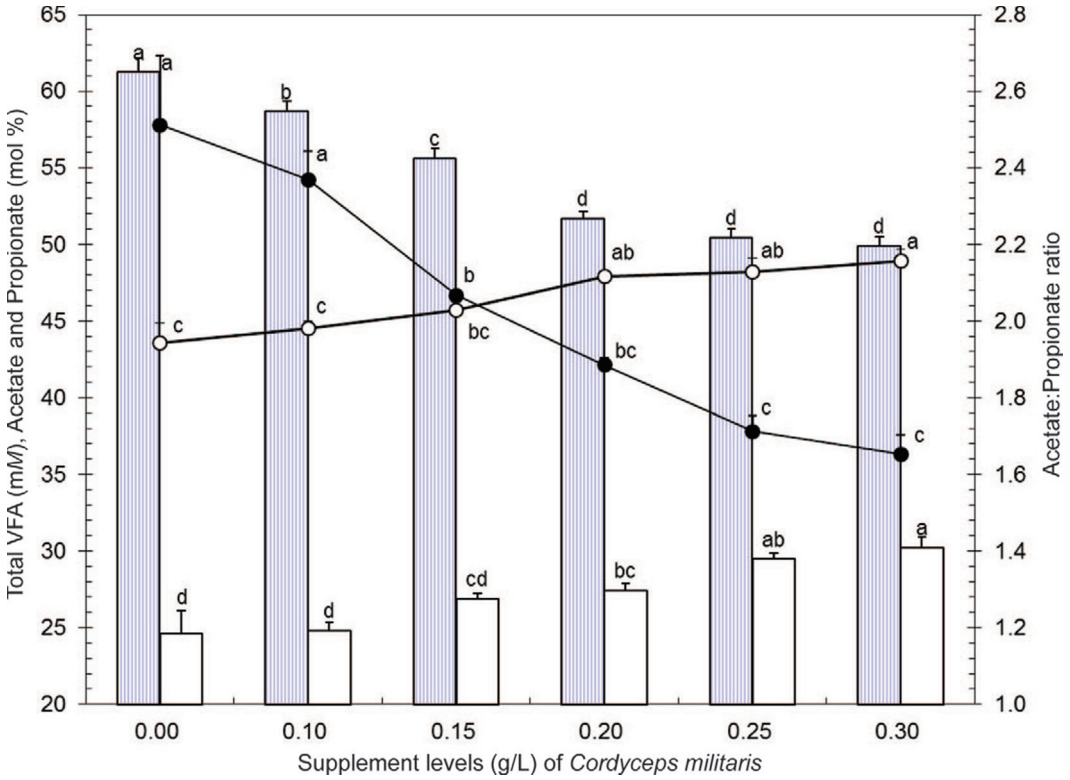

Figure 1. Influence of different doses of *Cordyceps militaris* on total VFA (○), and the molar proportion (%) of acetate (shaded bars), propionate (open bars), and acetate:propionate ratio (A:P ratio, ●) in supernatant of growing mixed rumen anaerobic microorganisms after a 24-h incubation. Lowercase letters indicate statistical significance; means (n = 3) with different letters are significantly different ($P < 0.05$). Color version available in the online PDF.

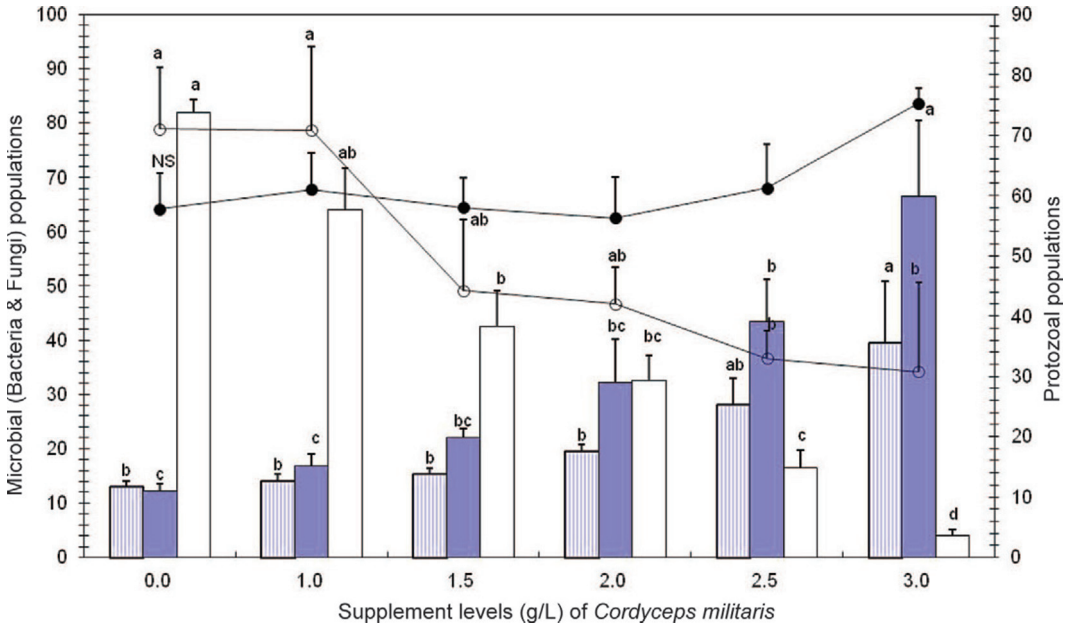

Figure 2. Influence of different doses of *Cordyceps militaris* on the populations of total bacteria ($\times 10^9$ cfu/mL, shaded bars), cellulolytic bacteria ($\times 10^6$ cfu/mL, solid bars), anaerobic fungi ($\times 10^3$ cfu/mL, open bars), live protozoa ($\times 10^2$ cfu/mL, ○), and dead protozoa ($\times 10^3$ cfu/mL, ●) in supernatant of growing mixed rumen anaerobic microorganisms after a 24-h incubation. Lowercase letters indicate statistical significance; means (n = 3) with different letters are significantly different ($P < 0.05$). Color version available in the online PDF.

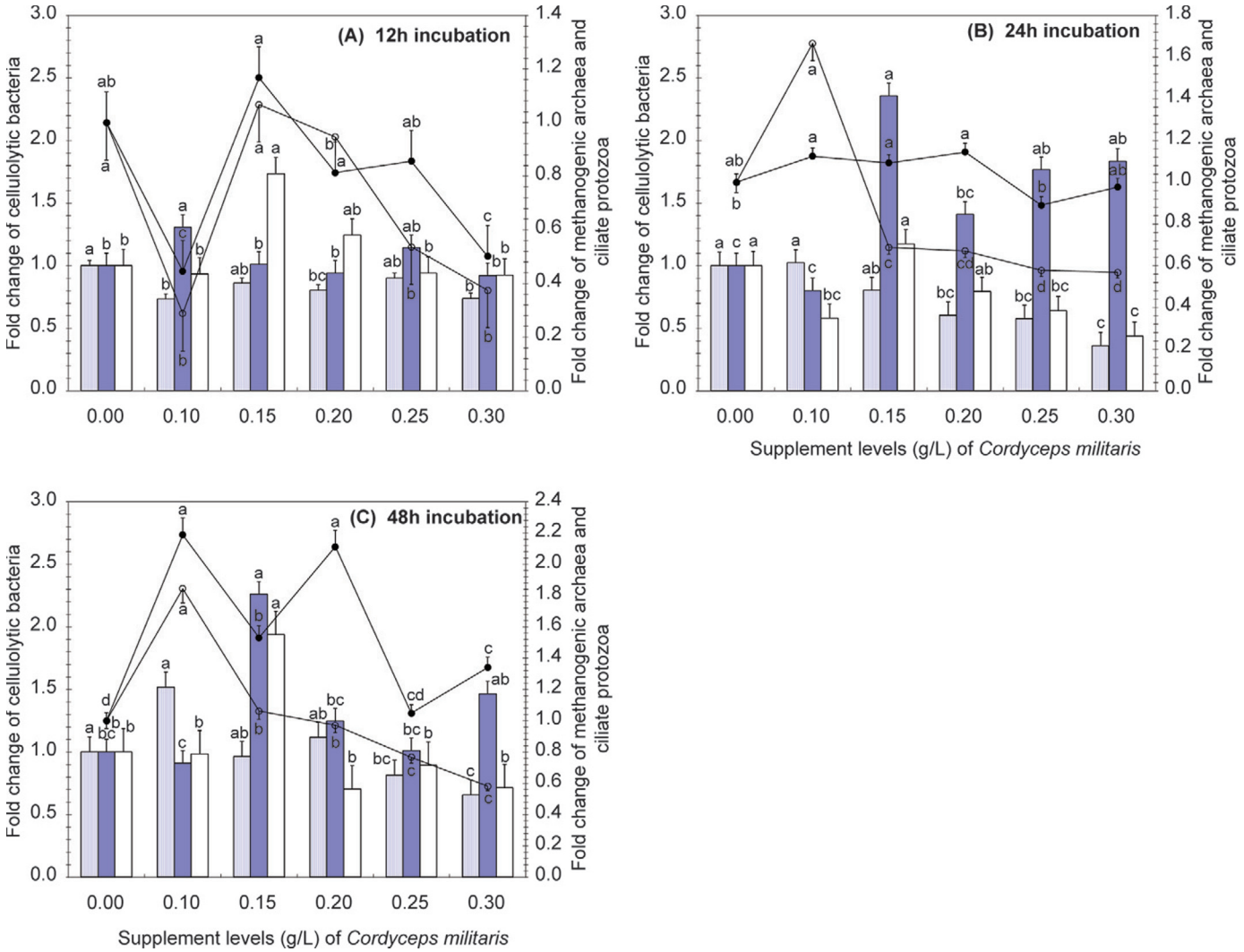

Figure 3. Influence of different doses of *Cordyceps militaris* on the relative quantification analysis of methanogenic archaea (●), ciliate protozoa (○), and cellulolytic bacteria: *Ruminococcus albus* (shaded bars), *Ruminococcus flavefaciens* (solid bars), and *Fibrobacter succinogenes* (open bars) in supernatant of growing mixed rumen anaerobic microorganisms after (A) 12-h, (B) 24-h, and (C) 48-h incubations. Lowercase letters indicate statistical significance; means (n = 3) with different letters are significantly different ($P < 0.05$). Color version available in the online PDF.

addition of *C. militaris*, except at the 0.25 g/L level, increased the abundance of methanogenic archaea.

DISCUSSION

In general, in vitro ruminal anaerobic microbial fermentation was strongly affected by the addition of dried *C. militaris*. The addition of *C. militaris* increased cumulative and potential gas production, but reduced production of methane and hydrogen gas. Supplementation with *C. militaris* appeared to accelerate the fermentation process, especially in the early stages of incubation, as shown by accelerated rates of cumulative gas production (Table 2). Total gas production was closely related to the digestion of fermentation substrates, VFA production, and microbial activity and growth (Getachew et al., 2004). In the present study, although we observed a lag time between gas production and the responses of pH and total VFA production, a positive correlation between gas production and total VFA production was found ($R^2 = 0.63$, $P < 0.001$). The increases in total gas production in response to the addition of *C. militaris*, which is highly nutritious, might be due to the increased activ-

ity of related microbes. It is true that the control vials contained less nutrients (N and carbohydrates) than did the treatment vials, but the supply of N in the control vials would seem unlikely to be limited during the early incubation periods, as shown by the similar ammonia-N concentrations between treatments (Table 4). Furthermore, ammonia-N concentration at 12 and 24 h was lower for the treatments than for the control. The difference in supply of carbohydrates would likely be minimal because of the high level of CP (76%) in *C. militaris*. This suggests that stimulatory responses to *C. militaris* might have been from an adverse effect on protozoan population and a positive effect on bacterial population rather than from differences in the supply of major nutrients. The numbers of total and cellulolytic bacteria were increased by the addition of *C. militaris* (Figure 2).

Methane produced by enteric fermentation in ruminants not only represents a severe loss of feed energy for the animals but also has an ecological impact. Therefore, reducing methane production could have significant economic and environmental benefits. In the present study, addition of *C. militaris* decreased methane production linearly from 24 to 72 h of incubation with a maximum reduction of 40.9% observed for the highest level of *C. militaris* at 24 h of incubation (Table 3). Kamra et al. (2008) screened 93 plant extracts for their potential to inhibit in vitro methanogenesis and ciliate protozoa using buffalo rumen liquor, and reported that 20 extracts abated methane production by more than 25%, accompanied by a sharp decline in methanogen numbers. Some plant species showing a more pronounced effect are rich in saponins (*Sapindus mukorossi*), tannins (*Terminalia chebula, Populus deltoids*, *Mangifera indica*, and *Psidium guajava*), or essential oils (*Syzygium aromaticum* and *Allium sativum*). In the RUMEN-UP project (Rumen Metabolism Enhanced Naturally Using Plants; http://www.rowett.ac.uk/rumen_up/index.html), potential candidates were selected from 500 different plant species based on their ability to inhibit methane production by 15 to 27% without a detrimental effect on total VFA production or feed digestibility. The plant species selected were the Italian plumeless thistle (*Carduus pycnocephalus*, 30% inhibition), the Chinese peony (*Paeonia lactiflora*, 8–53%), the European aspen (*Populus tremula*, 25%), the sweet cherry (*Prunus avium*, 20%), goat willow (*Salix caprea*, 30%), English oak (*Quercus pedunculata*, 25%), and Sikkim rhubarb (*Rheum nobile*, 25%). The application of these candidate species to ruminant livestock is still in the early stage and many points still need to be clarified (Kobayashi, 2010).

Our findings cannot be directly compared with numerous methane-suppressing agents reported in the literature because this is the first study to show that *C. militaris* can suppress methane emission. However, the modes of action of *C. militaris* appear similar to those of monensin and secondary plant metabolites (saponins) because the reduction of methane production in response to the addition of *C. militaris* was accompanied by a decrease in live protozoan population (Figure 2) and abundance of ciliate protozoa (Figure 3). It has been reported that monensin and saponin affect methanogens indirectly by suppressing ciliate protozoa (Hino et al., 1993; Lila et al., 2003). Rumen ciliate are known to provide hydrogen as a substrate for methanogens (Stumm and Zwart, 1986; Ushida et al., 1997), and methanogenic archaea are metabolically correlated with ciliate protozoa (Stumm et al., 1982; Newbold et al., 1995). Therefore, elimination of ciliate protozoa from the rumen leads to reduced methane emission (Jouany et al., 1981; Itabashi et al., 1984; Ushida et al., 1986). But it has been reported that the response of methane production to saponin in in vitro could be affected by saponin source and dose level (Hess et al., 2003; Beauchemin et al., 2008), and also by the potential adaptation of the rumen microflora and ruminal fiber digestion in vivo (Lu and Jorgensen, 1987; Holtshausen et al., 2009).

In the present study, a substantial reduction in methane production did not result in a corresponding decrease in the abundance of methanogenic archaea (Figure 3), as was observed in the study of Hess et al. (2003). It has been reported that decreases in methanogen populations may not necessarily lead to a reduction in methane production, at least within a short period of time (Zhou et al., 2011). The discrepancy between the production of methane and the dynamics of the methanogen population might be partly attributable to the insensitivity of some ruminal methanogens to *C. militaris*.

In the present study, although the adverse effects of *C. militaris* on protozoa were similar to those of monensin and saponin, the increase in numbers of cellulolytic bacteria resulting from addition of *C. militaris* (Figure 2) was different from increases due to additions of monensin and sarsaponin, in which cellulolytic bacteria numbers were reduced. It has been suggested that the main reason for the methane-suppressing effects of sarsaponin might be the inhibition of H_2-producing bacteria such as cellulolytic bacteria (Wang et al., 2000). In the present study, *C. militaris* increased *R. flavefaciens*, whereas *F. succinogenes* and *R. albus* were decreased (Figure 3). The reasons for the different responses

within cellulolytic bacteria populations to the addition of *C. militaris* are not clear.

It is also interesting to note that total VFA increased but ammonia-N concentration decreased as the supplementation level of *C. militaris* increased (Table 4). Volatile fatty acids are the end products of rumen microbial fermentation and represent the main supply of metabolizable energy for ruminants. Therefore, an increase in VFA production would be nutritionally favorable for the animal. In the present study, the addition of *C. militaris* increased total VFA in the culture fluid. Molar proportion of acetate and acetate:propionate ratio decreased ($P < 0.05$) and propionate increased as *C. militaris* increased (Figure 1). Similar results were obtained for monensin (Chen and Wolin, 1979; Newbold et al., 1995) and sarsaponin (Lila et al., 2003), both of which shifted the proportions of VFA toward higher propionate and decreased acetate. The decreased acetate:propionate ratio reflects both the reduced production of methane and the redirection of hydrogen from methane to propionate (Demeyer and Van Nevel, 1975).

Reduced ammonia-N concentrations in the rumen are typical when protozoa are inhibited (Williams and Coleman, 1991). It has been reported that the ionophores that inhibit gram-positive bacteria and protozoa also decrease deamination (Van Nevel and Demeyer, 1977). Similar to this, in the present study, a linear reduction of the concentration of ammonia-N, coupled with a decreased protozoan population, was seen following addition of *C. militaris*.

CONCLUSIONS

Dried *C. militaris* has the ability to partly inhibit methane production in in vitro microbial fermentations. This compound stimulated mixed ruminal microorganism fermentation and a change in fermentation products, and it decreased methane and hydrogen gas production. Further research is necessary to establish the long-term efficacy of *C. militaris* to inhibit methanogenesis and improve animal performance.

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