Effects of Flavonoid-rich Plant Extracts on *In vitro* Ruminal Methanogenesis, Microbial Populations and Fermentation Characteristics

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ABSTRACT: The objective of this study was to evaluate the *in vitro* effects of flavonoid-rich plant extracts (PE) on ruminal fermentation characteristics and methane emission by studying their effectiveness for methanogenesis in the rumen. A fistulated Holstein cow was used as a donor of rumen fluid. The PE (*Punica granatum*, *Betula schmidtii*, *Ginkgo biloba*, *Camellia japonica*, and *Cudrania tricuspidata*) known to have high concentrations of flavonoid were added to an *in vitro* fermentation incubated with rumen fluid. Total gas production and microbial growth with all PE was higher than that of the control at 24 h incubation, while the methane emission was significantly lower ($p<0.05$) than that of the control. The decrease in methane accumulation relative to the control was 47.6%, 39.6%, 46.7%, 47.9%, and 48.8% for *Punica, Betula*, *Ginkgo*, *Camellia*, and *Cudrania* treatments, respectively. Ciliate populations were reduced by more than 60% in flavonoid-rich PE treatments. The *Fibrobacter succinogenes* diversity in all added flavonoid-rich PE was shown to increase, while the *Ruminoccocus albus* and *R. flavefaciens* populations in all PE decreased as compared with the control. In particular, the *F. succinogenes* community with the addition of Birch extract increased to a greater extent than that of others. In conclusion, the results of this study showed that flavonoid-rich PE decreased ruminal methane emission without adversely affecting ruminal fermentation characteristics *in vitro* in 24 h incubation time, suggesting that the flavonoid-rich PE have potential possibility as bio-active regulator for ruminants. (**Key Words:** Flavonoid-rich Plant, Methane Emission, Microbial Growth, Ruminal Fermentation, Rumen Anaerobic Microbes)

INTRODUCTION

Agricultural greenhouse gas emission, mainly methane from ruminants, has currently been recognized as an important issue worldwide as it is a driver for global warming and climate change. Methane eructated from ruminants is considered to be one of the most important contributors to global warming, imposing an environmental burden that cannot be ignored. Meanwhile, it represents a loss of 2% to 15% of the gross energy intake (Johnson and Johnson, 1995; Ellis et al., 2007). Animal nutritionists have been studying manipulation of the rumen microbial ecosystem to reduce methane emission without the adverse effects on rumen function. There is a need to identify feed additives to modify ruminal fermentation characteristics and increase the efficiency of feed utilization, thereby inhibiting the ruminal methanogenesis. In recent years, essential oils (Benchaar, 2007), plant secondary metabolites such as condensed tannins and saponins (Pen et al., 2006; Bhatta et al., 2009) and dietary lipids (Dohme et al., 2001) have arisen as attractive rumen modifiers to improve rumen microbial metabolism as well as inhibit methane production in ruminants. The positive effects of flavonoid-rich plant extracts (PE) on methane emission and methanogens population *in vitro* as well as *in vivo* have been examined (Patra et al., 2006; Bodas et al., 2008; Patra and Saxena, 2010; Oskoueian et al., 2013; Becker et al., 2014). In

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addition, flavonoid supplementation could improve ruminal fermentation of dairy cows with increasing milk yield (Theodorou et al., 1994), protecting ruminal acidosis (Balcells et al., 2012), reducing methane emission and changing microbial populations such as protozoa and methanogen (Baker, 1999).

The objective of this study was to evaluate the effect of flavonoid-rich PE on the growth of rumen microorganisms using quantitative real-time polymerase chain reaction (PCR) assay and *in vitro* rumen fermentation with respect to methane emissions.

MATERIALS AND METHODS

Preparation of plant extracts

Plant extracts were obtained from Plant Extract Bank at the Korea Research Institute of Bioscience and Biotechnology (Daejeon, Korea). Plants were collected from fields in Korea (Table 1). Each plant was cut into small pieces and dried naturally under shade. The dried plant (100 g) were extracted with 99.9% methyl alcohol (1 L) using ultrasonic cleaner (Branson Ultrasonics corporation, Danbury, CT, USA) at room temperature for 3 days. After extraction, the solutions were filtered and the solvents were evaporated under vacuum. Stock solutions (20 mg/mL) of the extract were dissolved in dimethyl sulfoxide (Sigma-Aldrich Chemical Co., St. Louis, Mo, USA) and diluted using culture media immediately before experiments.

Ruminal inoculum and *in vitro* incubation

A fistulated Holstein cow was used as a donor of rumen fluid. Timothy and commercial concentrate in the ratio of 60:40 were fed at 2% of body weight twice a day (09:00 and 17:00). Water and mineral-vitamin block were allowed *ad libitum*. The rumen fluid was collected from the fistulated Holstein cow before morning feeding. Rumen liquor was filtered through four layers of cheesecloth before mixing with buffer and was maintained at 39°C. Fifteen mililiters of rumen fluid-buffer mixture, comprising McDougall buffer (McDougall, 1948) and rumen liquor in the ratio of 2 to 1, was dispensed anaerobically into 50 mL serum bottles containing 0.3 g of timothy substrate and PE (5% of substrate). The serum bottles were filled with O_2-free N_2 gas capped with a rubber stopper and held in a shaking incubator (Jeio Tech, SI-900R, Daejeon, Korea; 120× rpm) at 39°C for 72 h. The *in vitro* experiment was evaluated in triplicate run for data analysis using 90 serum bottles (6 treatments×5 incubation times×3 replication) with a completely randomized design.

Gas production measurement and analysis of gas profiles and ruminal fermentation

At the end of incubation, total gas production was measured according to the assay outlined by Theodorou et al. (1994). A detachable pressure transducer and a digital readout voltmeter (Laurel Electronics, Inc., Costa Mesa, CA, USA) were used to measure the headspace gas pressure of fermenting cultures. For the total gas production measurement, the transducer was modified in a way that it could be linked to the inlet of a disposable Luer-lock three-way stopcock (Theodorou et al., 1994). Gas pressure in the headspace was read from the display unit after insertion of the hypodermic syringe needle through the butyl rubber stopper above the culture medium. The headspace gas in the serum bottle was collected for analyzing methane and hydrogen by gas chromatography (Agilent Technologies HP 5890, Santa Clara, CA, USA) conducted using a TCD detector with a Column Carboxen 1006PLOT capillary column 30 m×0.53 mm (Supelco). The culture was subsampled for the analysis of pH (Mettler-Toledo, MP230, Greifensee, Switzerland), volatile fatty acid (VFA) concentration and genomic DNA extraction. The VFA analysis was performed with a HPLC (High Performance Liquid Chromatography, Agilent-1200, Waldbronn, Germany) equipped with column (300 mm×7.8 mm I.d. MetaCarb 87H, Varian, Palo Alto, CA, USA). *In vitro* DM disappearance rate was estimated by the modified method of nylon bag digestion process. Briefly, after incubation, the nylon bag with substrate was washed twice in a water-bath equipped with Heidolphs Rotamax 120 (Heidolph Instrument, Nuremberg, Germany) at 100×rpm for 30 min. Washed nylon bags were then dried to a constant weight at 60°C. Dry matter disappearance was determined by weight difference before and after incubation in the serum bottle.

Microbial growth rate

Incubated samples taken from each fermentation period

Table 1. Technical information regarding flavonoid-rich plant extracts used in the experiment[a]

Stock No.	Botanical name	Scientific name	Family name	Part[b]
034-052	Pomegranate	*Punica granatum*	*Punicaceae*	L+S+F
015-042	Brich	*Betula schmidtii*	*Betulaceae*	S
019-076	Ginkgo	*Ginkgo biloba*	*Ginkgoaceae*	L
001-053	Camellia	*Camellia japonica*	*Theaceae*	L
026-020	Tricuspid cudrania	*Cudrania tricuspidata*	*Moraceae*	L

[a] Plant extracts were obtained from Plant Extract Bank (PEB) at Korea Research Institute of Bioscience and Biotechnology (Daejeon, Korea).
[b] L: Leaf, S: Stem, F: Flower.

were centrifuged at 3,000×rpm for 3 min to remove feed particles, and the supernatants were re-centrifuged at 14,000×rpm for 3 min to settle the pellets down. After that, sodium phosphate buffer (pH 6.5) was added to these precipitates and vortexed. Growth rates of total microbes were estimated as optical density (OD) values using spectrophotometer (Model 680, Bio-Rad Laboratories, Hercules, CA, USA) at 550 nm.

Quantitative polymerase chain reaction assays

DNA extraction: A high-speed reciprocal shaker which retains samples in screw-capped tubes containing silica beads was used for DNA extraction. Total nucleic acid was extracted from the incubated rumen samples by using the modified bead-beating protocol with the Soil kit (Macherey-nagel, Düren, Germany). Briefly 1.0 mL aliquot was taken from the incubated culture solution and was centrifuged at 3,000×rpm. Nucleic acid concentrations were measured by using a NanoDrop Spectrophotometer (Thermo Scientific, Wilmington, DE, USA).

PCR primers: The PCR primer sets (Table 2) used in this study for amplification of total bacteria, *Fibrobacter succinogenes*, *Ruminococcus albus*, *Ruminococcus flavefaciens*, methanogenic archaea, and ciliate protozoa were from the published reports (Koike and Kobayashi, 2001; Denman and McSweeney, 2005; Skillman et al., 2006; and Denman et al., 2007). All microbial data were analyzed for calculating relative expressions to total bacteria (Denman and McSweeney, 2006).

Quantitative Real-time PCR: Quantitative PCR assays for enumeration of microbes were performed according to the methods described by Denman and McSweeney (2006) and Denman et al. (2007) on a real-time PCR Machine (CFX96 Real-Time system, BIO RAD, Hercules, CA, USA) using the SYBR Green Supermix (QPK-201, Toyobo Co., LTD., Tokyo, Japan). The values of cycle threshold (Ct) after real-time PCR were used to determine fold change (number of fold difference) of different microbial population relative to control without additives. Abundance of these microbes was expressed by the equation: relative quantification = $2^{-\Delta Ct(Target)-\Delta Ct(Control)}$, where Ct represents threshold cycle. All quantitative (q) PCR reaction mixtures (final volume of 20 μL) contained forward and reverse primers, the SYBR Green Supermix and DNA template. A negative control without the template DNA was used in every qPCR assay for each primer. The PCR amplification of the target DNA, included the annealing and the extension temperature, was conducted following the references in Table 2.

Total polyphenol and total flavonoid concentration

Total polyphenol concentration: Total polyphenol content in the PE was determined with Folin-Ciocalteu reagent using modified method by Velioglu et al. (1998) in a 96 well plate. Reaction mixture consisted of 10 μL of PE, 180 μL of Na_2CO_3 (2%), 10 μL of Folin-Ciocalteu reagent (1:1 with water) and 10 μL distilled water. Total polyphenolic content in PE was estimated as OD values using enzyme-linked immunosorbent assay (ELISA) reader (Spectra Max reader M5, Molecular Devices, Sunnyvale, CA, USA) at 750 nm after 30 min incubation (37°C). The standard calibration plot was generated at 750 nm using known concentration of gallic acid.

Total flavonoid concentration: The aluminum chloride method modified by Jia et al. (1999) was used for the determination of total flavonoid content of PE. Aliquots of PE solutions were taken and made up the volume 100 μL with methanol. Then, 7.5 μL of NaNO (5%), 15 μL of $AlCl_3$ (10%), 100 μL of NaOH (1 M) and 25 μL of distilled water were added sequentially. Total flavonoid content in PE was estimated as OD values using ELISA reader (Spectra Max reader M5, Molecular Devices, Sunnyvale, CA, USA) at 510 nm after 30 min incubation. The standard calibration plot was generated at 510 nm using known concentration of catechin.

Table 2. PCR primer sets for real-time PCR assay

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

PCR, polymerase chain reaction.
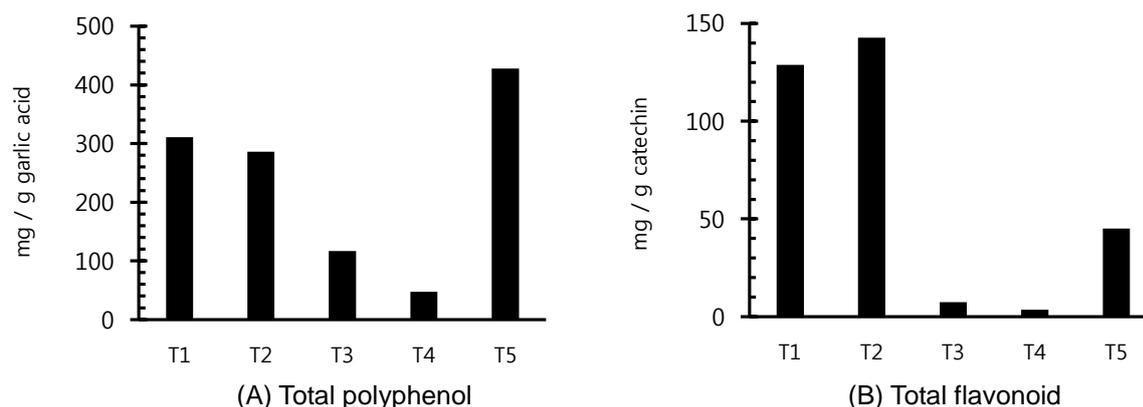
Figure 1. Concentration of total polyphenol (A) and total flavonoid (B) in flavonoid-rich plant extracts used in the experiment (T1, Pomegranate; T2, Birch; T3, Ginkgo; T4, Camellia; T5, Cudrania tricuspidata).

Statistical analysis

Data were analyzed using the general linear model procedure of the Statistical Analysis System Institute, Inc. (SAS Institute, 2002). The effects of PE on total gas production, gas profiles, pH, VFA, and microbial growth were compared to the controls and significant differences between treatment means were examined using Duncan's multiple comparison tests. A $p<0.05$ was considered to indicate statistical significance.

RESULTS AND DISCUSSION

***In vitro* ruminal fermentation characteristics**

The concentration of total polyphenol and total flavonoid in PE used in this experiment is shown in Figure 1. In particular, the concentration of total flavonoid was higher in Pomegranate and Birch extracts compared with others. The *in vitro* ruminal fermentation profiles are shown in Table 3. pH did not significantly show a difference ($p< 0.05$) with the addition of all PE except at 72 h incubation time. Our study supports the previous studies that pH was not significant different in *in vitro* ruminal incubation with flavonoids such as flavone, myricetin, naringin, catechin, rutin, quercetin and kaempferol (Oskoueian et al., 2013) as well as flavonoid, tannin and essential oil (Bodas et al., 2008). The tVFA concentrations were decreased or increased by flavonoid-rich PE according to incubation times (6 h, 12 h, 24 h, 48 h, and 72 h) but were not affected

Table 3. Effects of flavonoid-rich plant extracts on ruminal fermentation characteristics during 72 h incubation

	Treatments						SEM	p-value
	Control	Pomegranate	Birch	Ginkgo	Camellia	Cudrania tricuspidata		
	--- pH ---							
6 h	7.43[a]	7.31[a]	7.39[a]	7.45[a]	7.47[a]	7.52[a]	0.07	0.061
12 h	7.07[a]	7.06[a]	7.04[a]	7.08[a]	7.15[a]	7.15[a]	0.04	0.143
24 h	6.67[a]	6.71[a]	6.71[a]	6.69[a]	6.69[a]	6.71[a]	0.04	0.637
48 h	6.45[a]	6.46[a]	6.47[a]	6.47[a]	6.52[a]	6.49[a]	0.05	0.523
72 h	6.48[b]	6.47[b]	6.68[a]	6.65[a]	6.74[a]	6.66[a]	0.07	0.002
	--- tVFA (mM) ---							
6 h	30.96[a]	37.02[a]	30.66[a]	32.46[a]	30.90[a]	28.74[a]	4.73	0.424
12 h	37.99[b]	39.66[ab]	39.09[b]	40.05[ab]	38.27[b]	42.28[a]	1.45	0.037
24 h	64.08[a]	60.46[a]	59.93[a]	61.72[a]	62.49[a]	62.16[a]	1.87	0.168
48 h	78.21[a]	73.37[abc]	74.79[ab]	69.03[bc]	67.43[c]	70.87[bc]	3.77	0.041
72 h	75.80[a]	76.05[a]	64.92[ab]	66.36[ab]	57.51[b]	70.21[a]	6.49	0.033
	--- DM degradability (%) ---							
6 h	22.61[a]	22.17[a]	21.91[a]	22.10[a]	21.90[a]	20.58[b]	0.57	0.018
12 h	25.94[a]	25.59[a]	24.18[a]	24.65[a]	23.90[a]	27.67[a]	2.83	0.617
24 h	31.19[a]	30.67[a]	30.90[a]	29.85[a]	29.72[a]	28.50[a]	1.10	0.096
48 h	40.69[a]	41.88[a]	41.78[a]	39.65[a]	41.03[a]	40.80[a]	1.29	0.362
72 h	41.59[b]	46.17[a]	32.82[c]	41.78[b]	34.72[c]	43.04[b]	1.57	0.000

SEM: standard error of the mean; tVFA, total volatile fatty acids; DM, dry matter.

[a-c] Means in the same row with different superscripts differ significantly ($p<0.05$).

Table 4. Effects of flavonoid-rich included plant extracts on gas production and gas profiles during 72 h incubation

	Treatments						SEM	p-value
	Control	Pomegranate	Birch	Ginkgo	Camellia	Cudrania tricuspidata		
----- Total gas (mL/g DM) -----								
6 h	142.9[ab]	143.0[ab]	143.7[a]	144.0[a]	144.0[a]	142.0[b]	0.46	0.061
12 h	170.3[b]	179.1[a]	181.1[a]	179.2[a]	177.1[a]	175.5[ab]	2.02	0.033
24 h	218.9[a]	221.0[a]	220.3[a]	220.4[a]	220.4[a]	219.5[a]	2.33	0.989
48 h	258.5[b]	272.2[a]	237.8[c]	277.9[a]	245.1[c]	275.5[a]	3.97	< .0001
72 h	274.3[b]	294.9[a]	237.8[c]	278.1[b]	245.1[c]	276.8[b]	3.18	0.006
----- CH_4 (mL/g DM) -----								
6 h	1.70[a]	1.63[a]	2.09[a]	1.57[a]	1.60[a]	1.47[a]	0.26	0.610
12 h	6.24[a]	6.25[a]	5.46[a]	5.59[a]	5.63[a]	5.47[a]	0.45	0.632
24 h	15.87[a]	8.31[b]	9.59[b]	8.46[b]	8.27[b]	8.12[b]	1.35	0.010
48 h	29.90[ab]	22.81[ab]	16.99[b]	32.91[a]	22.61[ab]	35.70[a]	5.20	0.170
72 h	39.64[ab]	44.96[a]	30.16[c]	40.27[ab]	35.82[bc]	37.69[abc]	2.36	0.017
----- CO_2 (mL/g DM) -----								
6 h	19.73[a]	16.55[ab]	18.90[ab]	15.42[ab]	16.04[ab]	14.81[b]	1.72	0.384
12 h	44.08[a]	52.74[a]	48.39[a]	49.05[a]	49.75[a]	48.43[a]	3.37	0.643
24 h	77.47[a]	59.12[a]	74.65[a]	64.28[a]	67.89[a]	62.48[a]	9.23	0.705
48 h	102.1[ab]	109.0[ab]	74.99[d]	93.65[bc]	78.14[cd]	116.6[a]	5.41	0.001
72 h	104.4[a]	115.6[a]	103.9[a]	110.5[a]	107.81[a]	100.5[a]	6.30	0.613

SEM, standard error of the mean; DM, dry matter.

[a-d] Means in the same row with different superscripts differ significantly (p<0.05).

significantly as compared to control. Although dry matter (DM) disappearance was not significantly different except with the addition of *Cudrania tricuspidata* at 24 h incubation and pomegranate at 72 h incubation (p<0.05), DM disappearance in added flavonoid-rich PE was lower than that of the control at 6 h, 12 h, and 24 h incubation. The effect of flavonoid-rich PE on gas production and gas profiles is shown in Table 4. In particular, total gas production in added all PE was higher than that of the control at 24 h incubation, while the methane emission was significantly lower (p<0.05) than that of the control. This finding supports the finding that flavonoid-rich PE reduced methane emission by 4.7% to 14% after 24 h incubation

(Bodas et al., 2008). The decreased methane emission may be due to the changes in ciliate protozoan community (Figure 3B).

The *in vitro* change in microbial diversity in the rumen

Rumen bacterial growth with the addition of flavonoid-rich PE is shown in Figure 3. Although flavonoids are widely known to possess antifungal, antiviral and antibacterial activities (Cushnie and Lamb, 2005), the result of microbial growth under supplement of all PE was higher than that in control for 72 h incubation (Figure 2), which may be the cause of total gas production increase. The ciliate protozoa community with the addition flavonoid-rich
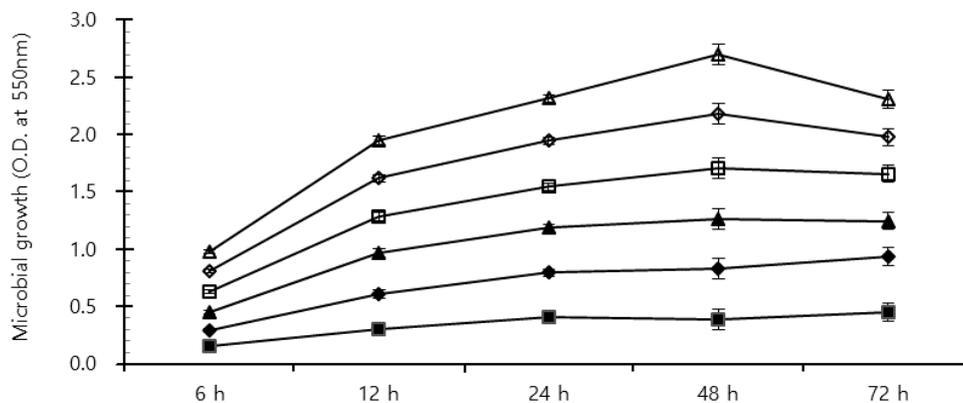
Figure 2. Effects of flavonoid-rich included plant extracts on growth rate of ruminal microbes in fermentation after time-scheduled batch incubation (■: control ♦: Pomegranate, ▲: Birch, □: Ginkgo, ◊: Camellia, Δ: Cudrania tricuspidata).
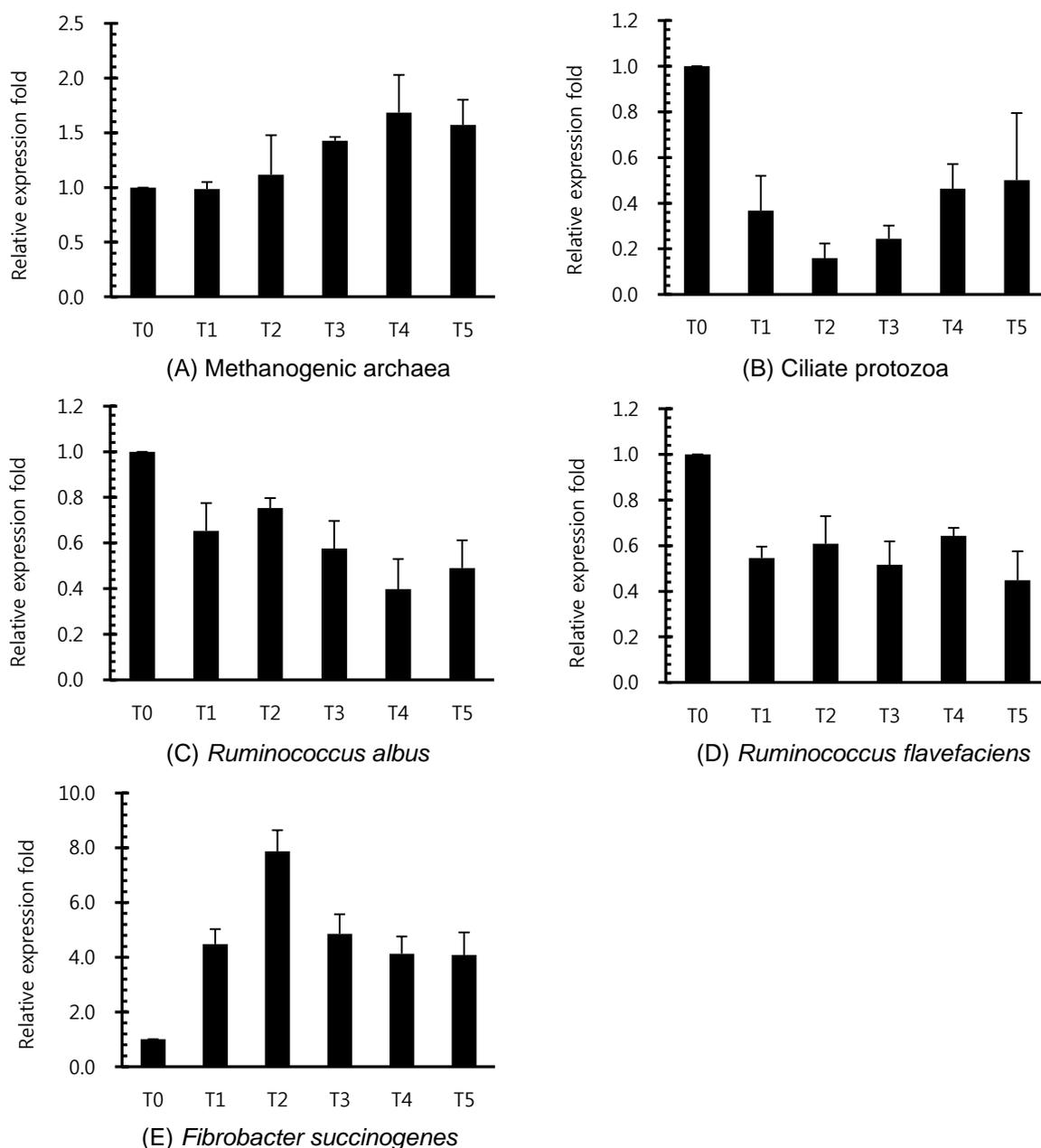
Figure 3. Relative quantification analysis of rumen microorganism populations *in vitro* ruminal fermentation by the addition of different plant extracts after 24 h incubation (T0, Control; T1, Pomegranate; T2, Birch; T3, Ginkgo; T4, Camellia; T5, Cudrania tricuspidata).

PE was decreased more than that of the control (Figure 3A). Patra et al. (2006) reported that extracts containing phenolics decreased the ruminal methane emission and protozoa count although they appeared not effective against ruminal methanogenesis. This study also showed that ciliate protozoan populations were reduced by more than 60% in flavonoid-rich PE treatments. Ciliate protozoa are an important key in methanogenesis in the rumen as methanogens attach to their surface. Flavonoid-rich PE reduced the ciliated-associated methanogens population and hence decreased the methane emission. Patra and Saxena

(2010) reported that flavonoids gave direct effects against methanogens, and reduced protozoa related with ruminal methanogenesis. The *Fibrobacter succinogenes* diversity under all flavonoid-rich PE was shown to increase, while the *Ruminococcus albus* and *Ruminococcus flavefaciens* populations under all PE decreased as compared with control. In particular, the *F. succinogenes* community with the addition of Birch extract increased to a greater extent than that of others (Figure 3C, 3D, and 3E). *R. albus*, one of ruminal fibrolytic bacteria, is a very promising bacteria to produce hydrogen (H_2) from energy forage, with the

potential of utilizing the cellulosic and hemicellulosic biomass (Ntaikou et al., 2008). Latham and Wollin (1977) reported that succinic acid is produced by *R. flavefaciens* culture as a major fermentation product with acetic and formic acids, H_2, and CO_2. H_2 is the critical concern to the microbial ecosystem in ruminants. H_2 produced during enteric fermentation is the precursor of methane emission from ruminants and the regulation of H_2 rather than methane is the key to control ruminant methane emission. The formation of propionate from succinate would result in a lower availability of H_2 for the methanogenesis. The ruminal microbe population may show that PE with flavonoid influences ruminal methanogenesis in this study.

In conclusion, the results of this study indicate that flavonoid-rich PE appears to have a potential possibility as bio-active regulator for ruminants with decreasing ruminal methane emission. Future studies need to be aimed at finding a suitable effective dose of PE for inhibiting ruminal methanogenesis.

ACKNOWLEDGMENTS

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