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Reduce the Energy Loss in Ruminant; Using *Raphanus Sativus* Extracts to Mitigate Methane Emission*

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반추동물의 에너지 손실을 줄이기 위한 연구; 무 추출물을 이용한 메탄 손실 억제

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This study was conducted to evaluate *Raphanus sativus* extracts to methane reduction in rumen. Five different levels of *R. sativus* extracts were used to investigate the most effective dosing level for the decrease of methane production in the rumen. The rumen fluid was collected from a cannulated one Hanwoo cow (BW=450±30 kg) consuming 600 g/kg timothy and 400 g/kg concentrate. On fermentation day, rumen fluid was collected at 2 hr postfeeding *R. sativus* extracts was dosed to achieve final concentration of 0, 1, 3, 5, 7, and 9% respectively, to fermentation bottles containing the mixture of rumen fluid and McDougall's buffer and 300 mg of timothy was added as a substrate. The fermentation was conducted for 3, 6, 9, 12, 24, 48 and 72 hr incubation time at 39℃ with shaking. *In vitro*

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ruminal pH values were measured normal range for ruminal fermentation. Dry matter disappearance was significantly higher ($p<0.05$) at 3 hr incubation time 1, 3 and 5% doses than that of control. The highest methane reduction was observed in 12 hr incubation time 5, 7 and 9%. The carbon dioxide emission was also significantly ($p<0.05$) lower than that of control at 12 hr incubation time 5, 7 and 9%. The total volatile fatty acid was no significant difference between control and all doses level at 12 and 24 hr incubation time. At 24 hr incubation time, the result of real-time PCR were indicated that *M. archea* was significantly lower ($p<0.05$) at all doses level comparing to that of control. In conclusion, R. sativus extracts were significantly decreased methane emission. *R. sativus* extracts were significantly lower ($p<0.05$) than that of control at 12 hr incubation time 5, 7 and 9% and no adversely effect in rumen pH, dry matter disappearance and total VFA.

Key words : *raphanus sativus, rumen fermentation, rumen methane emission*

Ⅰ. Introduction

Increased the concentration of greenhouse gases are inescapable consequence of global warming problem since the industrial revolution. As the earth's temperature has been increased, many natural phenomenon occurred such as in the antarctic glacier is melting and desert is bigger than the past. There are many greenhouse gases causing global warming but the methane and carbon dioxide is very representative factor of that. According to the IPCC, the methane is greater than that of carbon dioxide about 21 times impact on atmosphere (IPCC, 2013). That is why figuring out the way of decreasing the methane emission is important problem in study.

Ruminants account for more than 40% of the methane emissions from agriculture, and methane is all produced by enteric fermentation or manure from ruminants (Key and Tallard 2012). However, in this process ruminants loss 2~12% of feed energy (Moss et al. 2000). Reducing the methanogenesis from ruminants will not only reduce the loss of feed efficiency, but also have the effect of slowing the global warming.

Scientists around world have been doing a lot of research to mitigate methane emission from ruminants. Many experiments are conducted to decrease rumen methanogenesis by using a plant extracts as natural feed additives. Several plants included saponin and condensed tannin which is familiar substances to reduce methane (Lila et al., 2003; Puchala et al., 2005; Zhou et al., 2011). Based on previous studies, research on plants that can be easily obtained from the environment. These plants are including agro by-products as feed for ruminants and it may recycle the waste that has been thrown away as garbage (seo et al., 2015). However, there was no study focusing on methane reduction by agro by-products.

Raphanus. Sativus is representative agro by-products in Korea. In 2016, the total amount of fall *R. sativus* production was approximately 401 tons. Most of the domestic consumption of *R. sativus* is used by kimchi or pickled radish what is processed food and it makes a lot of agro by-product. Belong in to root vegetable, *R. sativus* is made up of root, stem and leaf. *R. sativus* is based on excellent nutritional ingredients, it is the best product to enhance the function of feed. With more than 90% moisture, large amount of vitamin C, fiber, mineral and sodium are included. Moreover, many digestive enzyme are also included amylase, amidase and glycosidase in the *R. sativus*, it may help to raise feed efficiency very well (Cho et al., 2009). Crude protein also high quality compare to other forage, it will be great substitution for concentrated feed. *R. sativus* contains flavonoid 'kaempferol' which has been known to inhibit methanogen growth in rumen efficiently (Ryu, 1999; oskoueian et al, 2013). Previous researches by *in vitro* experiments with *R. sativus* showed only digestibility evaluation and gas production in raw materials and in ruminants (Aderbal et al., 2015; Jeon et al., 2016). Therefore, the objective of this study is searching for the level of *R. sativus* extracts level to reduce methane emission and rumen fermentation characteristic.

Ⅱ. Materials and Methods

1. Preparation of *Raphanus sativus* extract

R. sativus 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. Each plant was cut into small pieces respectively and completely dried under the shade. The dried plants (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.

2. *In vitro* fermentation design

Rumen-cannulated one Hanwoo cow (BW=450±30 kg) was used as rumen fluid donors and provided with ad libitum access to water and a mineral-vitamin block. Timothy and commercial

concentrate ratio (60:40, w/w) were fed twice daily at 09:00 and 17:00, equivalent to 2% of body weight. The rumen fluid collected before morning feeding was strained through four layers of cheese gauze, and the rumen contents were diluted by addition of McDougall's buffer (McDougall, 1948) and maintained at 39°C. All experimental protocols used in this research were approved use committee of Gyeongsang National University (Jinju, Gyeongsangnam-do, Korea).

Dietary doses were as follows: Control (basal diet without *R. sativus* extract); *R. sativus* 1, 3, 5, 7 and 9% as basis of substrate (timothy). Table 1-1, Table 1-2 is showed information of chemical composition diets used in experiment roughage and concentrate.

15 mL of rumen fluid : McDougall's buffer mixture (1:2, v/v) was dispensed into 50 mL serum bottles containing 300 mg of timothy for Control and *R. sativus* extract for doses (3 μL for 1%, 9 μL for 3%, 15 μL for 5%, 21 μL for 7% and 27 μL for 9%). The serum bottles were sealed under anaerobic conditions and capped with a butyl rubber stopper with an aluminum cap and then incubated in a gently shaking incubator (Jeio Tech, SI-900R, Daejeon, Korea; 120 × rpm) at 39°C for 72 hr. The *in vitro* fermentation experiment was evaluated in triplicate for data analysis, using 126 serum bottles for 7 incubation times of each dose according to a completely randomized design.

Table 1-1. Chemical composition of diet used in experiments and feedstuff used as a substrate for *in vitro* incubation

Feedstuff	Chemical composition (% DM)						
	Moisture (%)	Crude protein (% DM)	Ether extracts (% DM)	Crude fiber (% DM)	Crude ash (% M)	NDF[1] (% M)	ADF[2] (% M)
Timothy	8.87	13.37	2.25	21.87	8.62	53.18	30.57

NDF[1] : Neutral detergent fiber.
ADF[2] : Acid detergent fiber.

Table 1-2. Chemical composition of diets used in experiment

Feedstuff	Chemical composition (% DM)						
	Crude protein	Crude fat	Crude fiber	Crude ash	Ca	P	TDN[1]
Concentrate	12.0	1.50	15.0	12.0	0.75	0.90	69.0

Ca : Calcium
P : Phosphorus
TDN[1] : Total Digestible Nutrients

3. Analysis of gas profiles and ruminal fermentation characteristics

Total gas production was measured according to the method that the head space gas pressure is to be measured using a detachable pressure transducer. A digital readout voltmeter (Laurel Electronics, Inc., Costa Mesa, CA, USA) after removing serum bottles from a shaking incubator (Theodorou et al., 1994). During gas profiling 72 hr, gas pressure in the headspace above the culture medium was read from the LED display unit after insertion of a syringe needle. Next another outlet was connected to a gas chromatograph (HP 5890, Agilent Technologies, Santa Clara, CA, USA) using a TCD detector with a Carboxen-1006 Plot capillary column (30 m × 0.53 mm, Supelco, Bellefonte, PA, USA) for detection of methane, carbon dioxide.

Serum bottles were uncapped and the culture medium was subsampled for pH analysis (MP230, Mettler-Toledo, Columbus, Ohio, USA) and volatile fatty acid (VFA) concentration. Take a measurement for VFA, culture medium was centrifuged at 12,000 × rpm for 3 min and then supernatants were filtrated using a 0.2 μm disposable syringe filter (Whatman Inc., Clifron, JN, USA). VFA analysis was implemented with a high performance liquid chromatography (HPLC, Agilent-1200, Waldbronn, Germany) using a UV/VIS detector with a MetaCarb 87H column (300 mm × 7.8 mm, Varian, Palo Alto, CA, USA).

In vitro DM disappearance rate was determined using the nylon bag digestion process (Ørskov et al., 1980). After incubation, the nylon bag containing the substrate was washed thrice in a water-bath equipped with a Heidolphs Rotamax 120 (Heidolph Instrument, Nuremberg, Germany) at 100 × rpm for 20 min (3 replicates) and then oven dried at 65°C to a constant weight. DM disappearance was defined as weight loss before and after incubation in the serum bottle.

4. Quantitative Real-Time PCR

To extract genomic DNA from rumen fluid, samples were mixed with ceramic and silica beads in a high speed reciprocal shaker (TissueLyser; QIAGEN, CA, USA). Total nucleic acid of the incubated rumen samples were purified with the modified bead-beating method using QIAamp DNA mini kit. Briefly, 1 mL aliquot was taken from the 30 mL incubated rumen sample and was centrifuged at 3,000 × rpm for measuring nucleic acid concentration using a NanoDrop spectrophotometer (Thermo Scientific, Wilmington, DE, USA).

The PCR primer sets used in this study for amplification of general bacteria (Denman et al., 2005), ciliate protozoa (Skillman et al., 2006), methanogenic archaea (Denman et al., 2007), *Fibrobacter succinogenes* (Denman and McSweeney, 2006), *Ruminococcus albus* (Koike and

Kobayashi, 2001) and *R. flavefaciens* (Denman and McSweeney, 2006) were from previously published reports.

Quantitative real-time PCR assays (CFX96 Real-Time system; BIO RAD, CA, USA) were performed with the SYBR Green Supermix (QPK-201, Toyobo Co., LTD., Tokyo, Japan), which was suggested by Denman and McSweeney (2005) and Denman et al. (2007). Abundance of these microbes was determined by the following equation: relative change in gene expression = 2–ΔCt (Target)–ΔCt (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. The PCR amplification of the target DNA, included the annealing and the extension temperature, was conducted following the same conditions of the PCR primer references.

5. Statistical analysis

Data were analyzed using the GLM procedure of the Statistical Analysis System Institute, INC. (SAS Institute, 2002). The effects of *R.sativus* extracts on pH, total gas production, DM disappearance, gas profiles and VFA profiles were compared to control and significant differences were compared to the controls and significant differences between doses means were examined using Duncan's multiple comparison tests. A $p<0.05$ was considered to be statistically significant.

Ⅲ. Results and Discussion

1. *In vitro* fermentation pH

The result of *in vitro* fermentation pH value is indicated in Table 2. All of the pH were stable at incubation time and *R. sativus* extracts doses. There was no significant difference in pH value at 3 and 72 hr incubation times compare to control. While at 6, 9 and 12 hr incubation times, 5, 7 and 9% doses have significantly higher ($p<0.05$) pH than that of control. At 24 hr 1, 3 and 9% doses level have significantly higher ($p<0.05$) than that of control. At 48 hr incubation time 3% dose has significantly higher ($p<0.05$) pH value compare to control. Using *in vitro* rumen incubation with a collected rumen fluid is effective way to mimic ruminal fermentation *in vivo*. (Tilly and Terry, 1963; Getachew et al., 1998). The normal range of rumen pH in ruminant is

6.0~7.0 (Ha et al. 2013).

Table 2. Effects of *Raphanus sativus* extracts on pH value

Incubation (hr)	Doses						SEM[1)]	P value	Mean
	Control	1%	3%	5%	7%	9%			
3	7.50^{a}	7.54^{a}	7.59 ^{a}	7.63^{a}	7.56^{a}	7.48^{a}	0.07	0.71	7.55
6	7.16^{c}	7.16^{c}	7.17^{c}	7.25^{b}	7.27^{ab}	7.31^{a}	0.02	0.00	7.22
9	7.06^{b}	7.11^{b}	7.12^{b}	7.19^{a}	7.21^{a}	7.21^{a}	0.02	0.00	7.15
12	7.07^{d}	7.08^{d}	7.10^{cd}	7.12^{bc}	7.14^{ab}	7.15^{a}	0.01	0.00	7.11
24	6.80^{c}	6.85^{ab}	6.85^{ab}	6.82^{bc}	6.80^{c}	6.86^{a}	0.01	0.01	6.83
48	6.53^{a}	6.37^{ab}	6.29^{b}	6.36^{ab}	6.35^{ab}	6.37^{ab}	0.07	0.27	6.38
72	6.15^{ab}	6.13^{ab}	6.09^{b}	6.19^{a}	6.21^{a}	6.20^{a}	0.03	0.09	6.16

^{abcd} Means with different superscripts in the same row differ significantly ($p<0.05$).

[1)] SEM : Standard error of the mean

2. Dry matter disappearance

The effect of various *R. sativus* extracts on the *in vitro* fermentation is shown in Table 3. At 3 hr incubation time 1, 3 and 5% doses were significantly higher ($p<0.05$) than that of control. Also, 6 hr incubation time 7% and 12 hr incubation time 1% doses were significantly higher ($p<0.05$) that of comparing to control. While 9, 24, 48 and 72 hr incubation times no significantly difference between any of *R. sativus* doses and that of control. Previous research have used raw material of *R. sativus* with forage and show that more addition of *R. sativus* with forages dry matter disappearance increase significantly (Jeon et al., 2016).

Table 3. Effects of Raphanus sativus extracts on *in vitro* dry matter disappearance (%)

Incubation (hr)	Doses						SEM[1)]	P value	Mean
	Control	1%	3%	5%	7%	9%			
3	17.15^{b}	18.83^{a}	19.17^{a}	19.13^{a}	18.19^{ab}	17.42^{b}	0.41	0.01	18.32
6	15.78^{b}	16.85^{ab}	17.07^{ab}	17.14^{ab}	17.73^{a}	16.07^{b}	0.46	0.09	16.77
9	18.78	18.03	18.43	18.21	17.96	17.97	0.65	0.93	18.23
12	18.53^{b}	20.03^{a}	18.40^{b}	18.28^{b}	18.36^{b}	18.50^{b}	0.42	0.09	18.68

Incubation (hr)	Doses						SEM[1)]	P value	Mean
	Control	1%	3%	5%	7%	9%			
24	25.83[a]	25.12[a]	25.53[a]	24.82[a]	24.75[a]	25.44[a]	0.35	0.28	25.25
48	35.37[a]	36.63[a]	37.06[a]	36.60[a]	37.17[a]	36.18[a]	0.64	0.43	36.50
72	39.60[a]	40.34[a]	39.62[a]	40.75[a]	39.70[a]	40.53[a]	0.78	0.82	40.09

[ab] Means with different superscripts in the same row differ significantly ($p<0.05$).
[1)] SEM : Standard error of the mean

3. Total gas production, methane emission and carbon dioxide

The effect of various *R. sativus* extracts on the gas profiles of total gas, methane and carbon dioxide emission are shown in Table 4.

The total gas production was significantly lower ($p<0.05$) than that of control at 3 hr incubation time 3, 5 and 7%. At 12 hr incubation time there was no significant difference compare to that of control. While 6 and 24 hr incubation time only 9% dose indicate significant lower ($p<0.05$) than that of control and At 9 hr incubation time 3, 5, 7 and 9% doses were observed exactly same result. However, at 48 hr incubation time was significantly higher ($p<0.05$) than that of control. Also at 72 hr incubation time was only 3% dose indicate significantly higher ($p<0.05$) than that of control.

The methane emission of 5% addition of *R. sativus* extracts significantly lower ($p<0.05$) than that of control at 3 hr incubation time 5% treatment. While 12 hr incubation time 5, 7 and 9% doses decreased methane emission significantly ($p<0.05$) compared to control. At 9 and 24 hr incubation times no significantly difference were observed in all doses compare to control. However, 6 hr incubation time 7 and 9% doses were significantly lower ($p<0.05$) compare to that of control. There was significant higher ($p<0.05$) at 48 hr incubation time 9% and 72 hr 3% comparing to that of control. Previous research showed that flavonoids tend to reduce methane production in rumen without decreasing DM disappearance and other ruminal fermentation characteristics. *R. sativus* extracts have component of the flavonoid is seems to have affected methane reduction in rumen (Oskoueian et al., 2013).

The carbon dioxide emission was no significant difference at 3, 9, 24 and 72 hr incubation times. At 12 hr incubation time 5, 7 and 9% doses were significantly lower ($p<0.05$) than that of control. While there was significant lower ($p<0.05$) in 6 hr incubation time only 9% dose. However, at 48 hr incubation time 7 and 9% doses there were significantly higher ($p<0.05$) comparing to that of control.

Table 4. Effects of *Raphanus sativus* extracts on gas profile

Incubation (hr)	Doses						SEM[1)]	P value	Mean
	Control	1%	3%	5%	7%	9%			
Total gas production (ml/g DM)									
3	162.18^{a}	154.90^{abc}	152.78^{c}	148.56^{c}	153.89^{bc}	161.23^{ab}	2.33	0.01	155.59
6	182.72^{a}	181.14^{ab}	183.14^{a}	178.50^{ab}	177.55^{ab}	173.80^{b}	2.23	0.08	179.47
9	195.92^{a}	191.28^{ab}	186.89^{b}	179.66^{c}	175.17^{c}	173.27^{c}	2.04	<.0001	183.25
12	189.64	185.26	187.00	185.52	185.52	184.20	2.04	0.52	186.19
24	218.31^{a}	212.87^{ab}	217.62^{a}	213.24^{ab}	216.41^{a}	208.23^{b}	2.01	0.03	214.45
48	253.53^{c}	260.29^{b}	270.64^{a}	265.88^{ab}	263.72^{b}	263.61^{b}	1.80	0.00	262.95
72	272.49^{b}	276.34^{b}	290.97^{a}	278.82^{b}	277.45^{b}	277.50^{b}	2.40	0.00	278.93
Methane emission (ml/g DM)									
3	1.76^{ab}	0.82^{bc}	0.73^{bc}	0.45^{c}	0.69^{bc}	2.54^{a}	0.37	0.01	1.16
6	9.56^{a}	7.41^{abc}	8.79^{ab}	7.07^{abc}	6.55^{bc}	6.10^{c}	0.79	0.06	7.58
9	14.60	8.79	11.84	10.71	9.10	8.88	1.01	0.01	10.76
12	16.06^{a}	10.29^{ab}	10.92^{ab}	6.40^{b}	8.27^{b}	7.19^{b}	1.88	0.04	9.86
24	18.13^{ab}	20.68^{a}	18.10^{ab}	11.02^{b}	15.77^{ab}	14.28^{ab}	2.11	0.08	16.33
48	36.49^{b}	39.13^{b}	47.82^{ab}	38.36^{b}	48.23^{ab}	59.64^{a}	4.08	0.01	44.95
72	79.32^{b}	90.85^{b}	103.89^{a}	86.88^{b}	81.33^{b}	81.06^{b}	4.00	0.01	87.22
Carbon dioxide emission (ml/g DM)									
3	59.95^{a}	49.20^{a}	51.27^{a}	46.14^{a}	51.94^{a}	54.44^{a}	4.89	0.49	52.16
6	100.97^{a}	101.32^{a}	99.79^{a}	89.15^{ab}	87.98^{ab}	76.04^{b}	6.30	0.09	92.54
9	120.14	89.59	106.53	88.10	86.64	90.79	9.50	0.14	97.40
12	111.22^{a}	108.85^{a}	100.82^{a}	59.90^{b}	67.91^{b}	62.36^{b}	6.10	<.0001	85.18
24	99.47	125.39	119.02	93.79	117.64	102.55	11.34	0.34	109.64
48	153.49^{c}	150.24^{c}	184.95^{bc}	166.87^{bc}	213.32^{ab}	259.47^{a}	16.15	0.00	188.06
72	294.39	295.00	314.70	291.91	292.60	304.80	11.28	0.67	298.90

^{abc} Means with different superscripts in the same row differ significantly ($p<0.05$).

[1)] SEM : Standard error of the mean

4. Total VFA

Table 5. is showing the volatile fatty acids of *R. sativus* extract. Total VFA, acetic acid,

propionic acid and A/P ratio there was no significantly difference between control and any of *R.sativus* doses at 12 and 24 hr incubation times. All of the VFA concentrations from *in vitro* ruminal fermentation are a great index of evaluation of nutritional feed quality (Makar, 2004).

Table 5. Effects of *Raphanus sativus* extracts on VFA

Incubation (hr)	Doses						SEM[1)]	P value	Mean
	Control	1%	3%	5%	7%	9%			
Total VFA (mM/g)									
12	60.40	59.19	59.38	58.88	60.08	58.68	1.39	0.94	59.44
24	72.69^{ab}	71.24^{b}	73.64^{a}	71.66^{b}	72.70^{ab}	71.84^{b}	0.49	0.04	72.30
Acetic acid (mM/g)									
12	40.20	39.25	38.99	39.26	39.69	39.10	0.72	0.84	39.42
24	48.17^{ab}	47.28^{b}	48.75^{a}	47.51^{b}	48.69^{a}	48.75^{a}	0.36	0.04	48.19
Propionic acid (mM/g)									
12	13.07	12.85	12.70	12.95	12.76	12.79	0.21	0.82	12.85
24	16.67^{abc}	16.37^{c}	16.96^{a}	16.52^{bc}	16.92^{ab}	17.00^{a}	0.13	0.02	16.74
A/P ratio									
12	3.08	3.05	3.07	3.03	3.11	3.06	0.03	0.56	3.07
24	2.89	2.89	2.87	2.88	2.88	2.87	0.03	0.99	2.88

^{abc} Means with different superscripts in the same row differ significantly ($p<0.05$).

[1)] SEM : Standard error of the mean

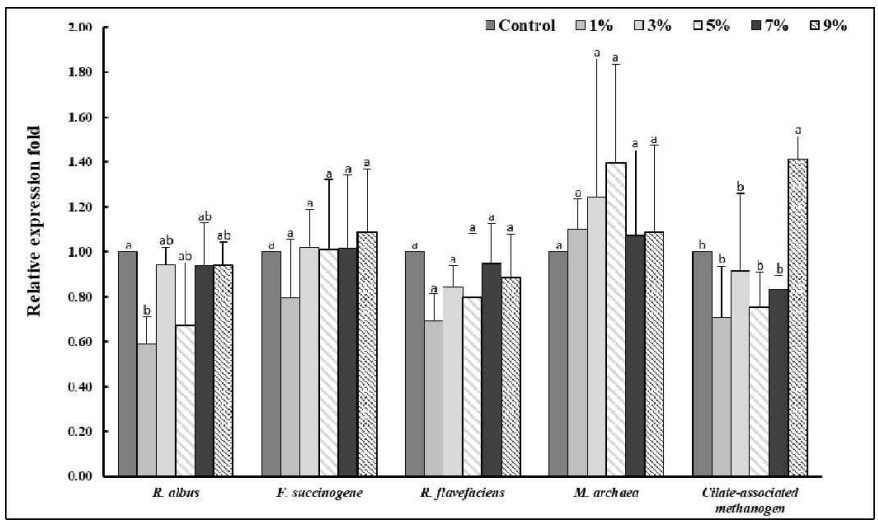
5. Ruminal microbial populations

Fig. 1, indicated the effects of *Raphanus. Sativus* extracts on the list of microbial populations in *in vitro* ruminal fermentation at 12 and 24 hr incubation time as implemented by PCR amplification of the target DNA for five microorganisms. There was no significant difference in the population of *F. succinogene*, *R. flavefaciens* and *M. archea* between all doses at 12 hr incubation time. There was significantly lower ($p<0.05$) than that of control in the population of *R. albus* at 12 hr incubation time 1% dose. However, *Ciliate-associated-methanogen* was significantly higher ($p<0.05$) than that of control in the population at 12 hr incubation time 9% doses.

At 24 hr, incubation time there was significant ($p<0.05$) reduction in the population of *M.*

archea all of the doses comparing to that of control. However, *F. succinogene* there was significantly lower than that of control at 1 and 5% doses. There was no significantly difference in the population of *R. albus*, *R. flavefaciens* and *Ciliate-associated-methanogen* compare to that of control.

(a) RT-RCR 12h



(b) RT-RCR 24h

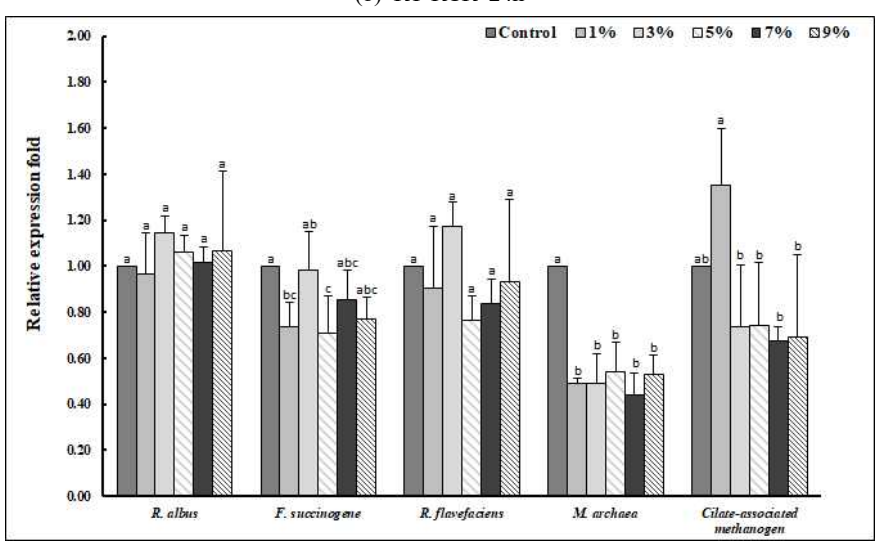


Fig. 1. Relative quantification analysis of rumen microorganism populations under *in vitro* ruminal fermentation by the addition of *Raphanus sativus* extracts after 12 h (a) and 24 h (b).

Ⅳ. Conclusion

Using agro by-product is helpful to reduce a lot of livestock feed expenses. This study is using *Raphanus sativus* extract to investigate the fermentation characteristics and search for the level of extract with high potential to inhibit methane emission. In addition, evaluate the function as feed additives. After *in vitro* evaluation, rumen pH value, dry matter disappearance and total VFA no adversely affect microbial fermentation in the rumen. The highest effect was observed in the 5% dose for 12 hr incubation time. Further studies are needed that previous studies have shown that among the component of *R. sativus* kaempferol which is content of flavonoid is effective in reducing methane emission. In addition, using raw material of *R. sativus* was significantly effective to lower ($p<0.05$) the total gas emission. However, there was no data about in rumen methane emission. Therefore, it is considered that an experiment to measure flavonoid content of *R.sativus* is needed and evaluate raw material of *R. sativus* effectiveness in rumen methane emission (Aderbal et al., 2015).

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