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Effect of Roughage to Concentrate Ratio and Plant Oil Supplementation on *In vitro* Fermentation End-Products

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Abstract: An *in vitro* study was conducted to evaluate effect of roughage to concentrate ratio (R:C) and coconut oil (CO) and wild almond seed oil (WO) supplementation on gas production, volatile fatty acid (VFA) concentration, methane production and dry matter disappearance. Completely randomized design was used for sixteen treatments. Treatments were 4 x 4 factorial arrangement where four of R:C ratios (100:0, 75:25, 50:50 and 25:75) and four of oil supplementation (un-supplement, 5% CO, 5% WO and 2.5% CO+2.5% WO). The potential extent of gas production was quadratically responded to R:C ratio ($p<0.01$) while gas production from all fractions of feed were suppressed by oil supplementation ($p<0.05$). Total VFA production, propionic acid proportion and calculated methane production were linearly increased with concentrate ratio; while oil supplementation decreased ($p<0.05$) these parameters especially when supplemented with wild almond seed oil. Dry matter disappearance at 24 h of incubation was increased with concentrate quantity but decreased by oil supplementation ($p<0.05$). It could be concluded that R:C ratio and seed oil remarkably influenced on fermentation end-product and gas production.

Key words: R:C, coconut oil, wild almond seed oil, gas production, volatile fatty acid, methane

INTRODUCTION

Methane is a greenhouse gas whose effect is estimated to be 25 times that of CO_2 based on equal molar amounts. Ruminant animals are one of the largest sources of methane emission with 81-92 million tons produced per year globally which is equivalent to 23-27% of total anthropogenic methane (IPCC, 2007). Methane produced during ruminal fermentation represents a loss of 2-15% of gross energy intake and thus decreases the potential conversion of digesta to metabolizable energy (Giger-Reverdin and Sauvant, 2000). Therefore, a reduction in methane emissions could increase BW gain of growing cattle or milk production of dairy cows, based on the energy balances reported by Nkrumah *et al*. (2006). The relationship between concentrate proportion in the diet and methane production is curvilinear (Sauvant and Giger-Reverdin, 2007) with a marked decrease in methane observed when dietary starch is higher than 40%. This has been assessed in young bulls by Martin *et al*. (2010), accordingly, a positive correlation between cellulolytic bacteria and methanogen (methanogenic microbe) in the rumen of different animal species (cattle, sheep, llamas, deer) has been shown (Morvan *et al*., 1996). Compared to diets containing 30% starch, a diet containing 45% starch decreased methane production by 56% without altering animal growth. On the other hand, fat inclusion in the diet causes a marked decrease in methane production by rumen fluid with the effect being at least partly governed by the fat source used (Machmuller *et al*., 2003). Supplementation of coconut oil shown decrease methane production in ruminants (Soliva *et al*., 2003; Jordan *et al*., 2006; Kanpukdee and Wanapat, 2008). In previous study Pilajun and Wanapat (2013) found that in *in vitro* ruminal fluid fermentation was improved by supplementation of coconut oil. Machmuller *et al*. (2003) stated that medium-chain fatty acid (MCFA) namely lauric acid (C12:0) and mylistic acid (C14:0) contained in oils had impacted on improving the fermentation. Wild almond (*Irvingia malayana*, Oliv. ex. A. Benn.) or in Thai krabok, is a tree grown widely in the tropical and subtropical areas, in addition wild almond seeds are consumable by the people. Yuangklang *et al*. (2011) found that ground wild almond seed can be used as an energy source at 10% in concentrate diet for sheep without any adversely affect on nutrient digestion. Wild almond seed oil is rich in C12:0 and C14:0 (Wongsuthavas *et al*., 2007) therefore, decreasing methane production could be obtained by this type of oil addition. Using of this oil it was found that fiber digestion, protozoal number and methane production were decreased, whereas the

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propionate production was increased in meat goats (Yuangklang *et al*., 2010). Panyakaew *et al*. (2008) also found that MCFA from coconut oil and wild almond seed oil did influenced on rumen methane production. The objective of the present study was to investigate the effect of concentrate proportion combined with coconut oil and wild almond seed oil supplementation on fermentation end-products in *in vitro* gas fermentation production.

MATERIALS AND METHODS

Experimental design: An *in vitro* study was conducted to evaluate effect of roughage to concentrate ratio and supplementation with coconut oil (CO) and Wild almond seed oil (WO) supplementation on fermentation end-products. Completely randomized design was used with 4 x 4 factorial arrangement where four of R:C ratios (100:0, 75:25, 50:50 and 25:75) and four schemes of plant oil supplementation (un-supplemented, 5.0% CO, 5.0% WO and 2.5% CO+2.5% WO supplemented); therefore, sixteen treatment combinations were used.

Gas fermentation production technique: The method used for *in vitro* fermentation based on the technique described by Menke *et al*. (1979). Two hundred milligrams of feed samples, with respective ratio of roughage and concentrate, were weighed into 100 mL bottle. Rice straw contained 2.4% of crude protein while cassava chip, rice bran, cottonseed meal and palm kernel meal were used in the concentrate mixture (14% crude protein). Cooking coconut oil and wild almond seed oil were carefully added in each treatment. Buffered mineral solution was prepared and placed on a magnetic stirrer at 39°C under continuous flushing with CO_2. Rumen fluid was collected before the morning feeding from two ruminally fistulated swamp buffaloes, fed with 75:25 rice straw as a roughage and a concentrate (14% CP and 78% TDN) ratio. Rumen fluid was taken and transferred into pre-warmed thermos flasks, combined, filtered through one layer of cheesecloth and flushed with CO_2. Forty mL of buffered rumen fluid were taken into bottle containing the feed samples. The bottles were placed in an incubator at 39°C for incubation.

Sample collection and analysis: The gas production was measured at, 1.5, 3, 6, 9, 12, 24, 36, 48, 72 and 96 h of incubation using glass syringe. Cumulative gas production data were fitted to the model of Orskov and McDonald (1979) as follows:

$$Y = a+b\,(1-e^{(-ct)})$$

where, a = the gas production from the immediately soluble fraction, b = the gas production from the insoluble fraction, c = the gas production rate constant for the insoluble fraction (b), t = incubation time, (a+b) = the potential extent of gas production and y = gas produced at time "t". Dry matter disappearance was measured at 24 h of incubation. The residues were washed three times in phosphate buffer (pH = 7.4) followed by centrifugation (2500 rpm, 10 min, 4°C). The pellets were dried at 100°C for 12 h for total dry matter determination. The fermented contents were sampled at 24, 48, 72 and 96 h of incubation, fixed in 2 M of H_2SO_4 for volatile fatty acids analyzed using HPLC according to Samuel *et al*. (1997). In addition, methane gas was calculated by using volatile fatty acid proportion according to equation of Moss *et al*. (2000):

$$\text{Methane } (CH_4) = 0.45C_2 - 0.275C_3 + 0.40C_4$$

where, C_2, C_3 and C_4 are proportions of acetate, propionate and butyrate, respectively.

Statistical analysis: Data were statistically analyzed using the General Linear Models (GLM) procedure in SAS application (SAS, 1996). Mean separations with a significant F ($p<0.05$) for treatment combination and effect of oil supplementation were compared by Duncan's New Multiple Range Test (DMRT) (Steel and Terrie, 1980), while R:C ration effect was compared using orthogonal polynomial.

RESULTS

The average of accumulate gas production in this study was 53.2 mL/treatment (Fig. 1 and 2). It showed that gas production accumulation was influenced by treatment which was increased with increasing concentrate proportion but slightly decreased by plant oil addition.

Gas production kinetic including the gas production from the immediately soluble fraction (a), the gas production from the insoluble fraction (b), the gas production rate constant for the insoluble fraction (c) and the potential extent of gas production (a+b), was affected by treatments ($p<0.05$); however, it was not found interaction effect ($p>0.05$) (Table 1). The gas production from the immediately soluble fraction was linearly decreased while the rate of gas production was increased with increasing level of concentrate ($p<0.01$). Moreover, the gas production from the insoluble fraction and the potential extent of gas production were quadratically responded to R:C ratio ($p<0.01$). Although the control (un-supplemented) resulted in highest of the gas production from the insoluble fraction and the potential extent of gas production, the rate of gas production was lowest ($p<0.05$) when compared with oil supplementation. Total gas production was significantly affected by R:C ratio ($p<0.05$) by linearly increased with increasing level of concentrate proportion. Moreover, addition of plant oils did decrease total gas production when compared with the control ($p<0.05$). Dry matter

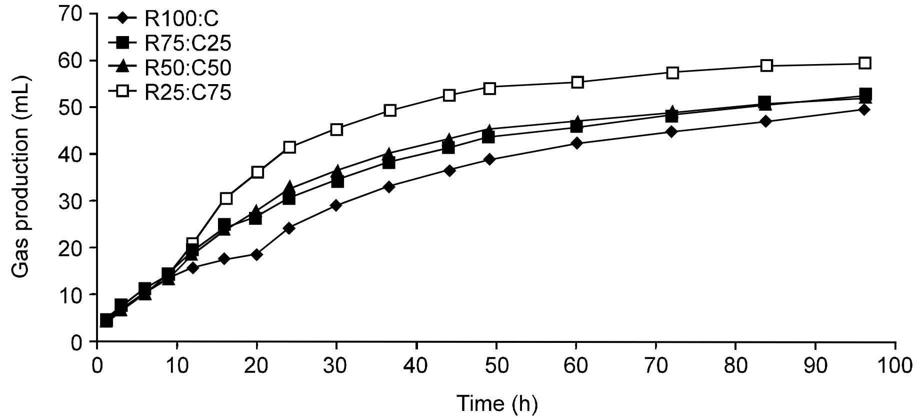

Fig. 1: *In vitro* gas production as affected by roughage (R) to concentrate (C) ratio

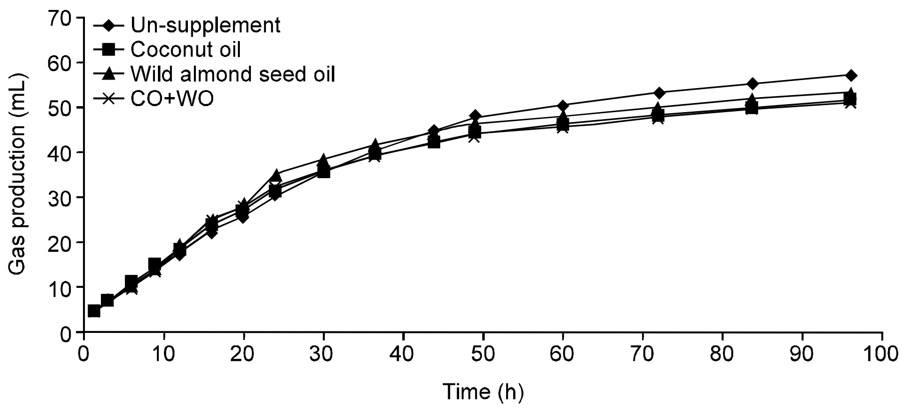

Fig. 2: *In vitro* gas production as affected by plant oil supplementation at 5% DM of substrate. CO+WO equal coconut oil to wild almond seed oil at 1:1 ratio

disappearance at 24 h of incubation was linearly affected by R:C ratio ($p<0.05$) but not with plant oil supplementations ($p>0.05$).

Effect of treatments on volatile fatty acid production was shown in Table 2. Total volatile fatty acid (TVFA) concentration and proportion of acetic acid (C_2), propionic acid (C_3) and butyric acid (C_4) were different among treatments in all of the sampling times ($p<0.05$). Increasing the concentrate proportion resulted in linearly increase of TVFA production and C_3 proportion; in contrast, proportion of C_2 was decreased. However, interaction of R:C ratio and oil supplementation was significant for volatile fatty acid production at 72 and 96 h of incubation. At 48, 72 and 96 h of incubation, TVFA concentration and C3 proportion were decreased by oil supplementation ($p<0.05$) which is opposite to the result of C_2. Wild almond seed oil adding showed the highest impact on volatile fatty acid production particularly when the incubation time was lengthen (72 and 96 h).

Methane production as calculated by using proportion of volatile fatty acid was changed by treatments ($p<0.05$), linearly decreased by increasing concentrate percentage ($p<0.05$). Although methane production (mole per mole of volatile fatty acid) was increased by oil supplementation ($p<0.05$), wild almond seed oil addition decreased methane production (mole per gram of substrate) when compared with the control ($p<0.05$). Besides, interaction of R:C ratio and plant oil supplementation on methane production at 96 h of incubation was found ($p<0.05$) (Table 3).

DISCUSSION

Gas production, *in vitro* digestion and volatile fatty acid concentration especially proportion of propionic acid were noticeably increased with increasing concentrate as the substrate. The result of this study agreed with our previous trial by Cherdthong *et al.* (2010), who found increasing total volatile fatty acid concentration and propionic acid percentage in the rumen of swamp buffalo fed with different quantity of concentrate. This could be due to concentrate that contained higher degradable carbohydrate fraction particularly starch than roughage. Calsamiglia *et al.* (2008) reported that high-concentrate diets tended to ferment toward propionate which agreed with Hungate (1966) who reported that

Table 1: Effect of R:C ratio and oil supplementation on 96 h gas production and dry matter disappearance using *in vitro* fermentation technique

Treatment[1]			Gas production kinetic[2]					
R:C	CO	WO	a	b	c	a+b	Total gas prod. (mL)	24 h DM Dis.
100:0	-	-	3.11	66.3	0.013	69.4	48.3	30.5
100:0	5.0	-	4.62	50.1	0.020	54.7	46.3	29.7
100:0	-	5.0	1.43	60.7	0.023	62.1	56.9	45.7
100:0	2.5	2.5	4.29	45.7	0.027	50.0	46.1	43.4
75:25	-	-	2.37	60.7	0.027	63.1	57.6	47.8
75:25	5.0	-	2.14	50.4	0.037	52.6	52.0	54.5
75:25	-	5.0	0.78	49.2	0.037	50.0	49.8	50.7
75:25	2.5	2.5	1.13	49.7	0.037	50.8	50.1	40.0
50:50	-	-	0.37	62.9	0.030	63.3	60.2	55.6
50:50	5.0	-	0.31	50.5	0.040	50.8	49.7	53.3
50:50	-	5.0	0.95	48.6	0.037	49.6	48.6	50.2
50:50	2.5	2.5	0.17	50.1	0.037	50.3	49.1	54.6
25:75	-	-	-1.09	67.0	0.037	65.9	62.7	53.9
25:75	5.0	-	-2.03	61.1	0.047	59.1	57.7	56.3
25:75	-	5.0	-2.25	61.0	0.050	58.8	58.0	56.6
25:75	2.5	2.5	-2.13	61.9	0.047	59.7	58.8	59.2
SEM	1.02	3.46	0.003	2.87	3.21	3.27		
Effect of R:C ratio								
100:0			3.37^{a}	55.7^{b}	0.021^{c}	59.1^{a}	49.4^{b}	37.3^{c}
75:25			1.61^{b}	53.0^{b}	0.034^{b}	54.1^{b}	52.4^{b}	48.2^{b}
50:50			0.45^{b}	52.5^{b}	0.036^{b}	53.5^{b}	51.9^{b}	53.4^{ab}
25:75			-1.88^{c}	62.7^{a}	0.045^{a}	60.9^{a}	59.3^{a}	56.5^{a}
Linear			**	**	**	ns	*	**
Quadratic			ns	**	ns	**	ns	*
Cubic			ns	ns	**	ns	ns	ns
Effect of oil supplementation								
Un-supplement			1.19	64.2^{a}	0.027^{b}	65.4^{a}	57.2^{a}	47.0
CO			1.26	53.0^{b}	0.036^{a}	54.3^{b}	51.4^{b}	48.4
WO			0.23	54.9^{b}	0.037^{a}	55.1^{b}	53.3^{b}	50.8
CW			0.87	51.8^{b}	0.037^{a}	52.7^{b}	51.0^{b}	49.3
RC*Oil interaction			ns	ns	ns	ns	ns	ns

Prod: Production, Dis: Disappearance, [1] R:C: roughage to concentrate ratio, CO: Coconut oil (5.0% of substrate)
WO: Wild almond seed oil (5.0% of substrate), CW, 2.5% CO and 2.5% WO mix
[2] a, the gas production from the immediately soluble fraction; b, the gas production from the insoluble fraction
c, the gas production rate; a+b, the potential extent of gas production
[a-c] Values on the same column and factor with different superscripts differed ($p<0.05$)
**$p<0.01$; *$p<0.05$; ns, non-significantly different; SEM, standard error of the mean

bacteria in the rumen would ferment the starch and soluble carbohydrate to volatile fatty acid mainly to propionic acid, or incorporated into their cells. Moreover, Hvelplund *et al.* (2009) found that the digestibility of starch in the rumen is valid for the range between 58 and 90%. Calculated methane production linearly decreased when percentage of concentrate increased and should be caused by increasing propionic acid proportion. The curvilinear relationship between concentrate proportion in the diet and methane production was found by Sauvant and Giger-Reverdin (2007) with a marked decrease in methane observed when dietary starch is higher than 40%. Agreed with Moss *et al.* (2000) who found methane was a good negative correlation with propionic acid (r^2 = 0.774) which is involved in hydrogen utilization by competing with methanogenesis (Sauer and Teather, 1987).

Plant oil supplementation decreased gas production and volatile fatty acid concentration but not in *in vitro* digestibility. These results agreed with our previous study (Pilajun and Wanapat, 2013) in which the reduction of gas production by coconut oil inclusion without adversely affect on digestion. However, Yuangklang *et al.* (2010) found that supplemental wild almond oil as a fat source reduced fibre digestion, whereas it increased the propionate production in meat goats. The depression following oil supplementation can most probably be attributed by the negative effect of medium-chain fatty acids (MCFA) on the fermentation. The MCFA in coconut oil and wild almond seed oil were small enough to be readily dissolved in the lipid phase, to penetrate and physically disrupt cell membranes and to inhibit enzymes involved in energy production and nutrient transfer, leading to reversible and irreversible changes that may lead to the death of the microbial cell (Machmuller, 2006). However, Palmquist (1994) reported that fiber digestion will be restricted when ruminants receive diets with a fat content higher than 7.0% of dry matter while only 5.0% of total fat used in the present study. Increasing methane production (per mole of volatile fatty acid) could be due to increased of acetic acid and butyric acid proportion when plant oils were added. Both of acetic acid and butyric acid proportions play the important role on methane synthesis in the equation of Moss *et al.* (2000), (+0.45 and +0.40,

Table 2: Effect of R:C ratio and oil supplementation on volatile fatty acid production using *in vitro* fermentation technique

Treatment[1]			24 h				48 h			
R:C	CO	WO	TVFA[2]	C2	C3	C4	TVFA	C2	C3	C4
100:0	-	-	57.1	78.3	9.9	11.9	72.0	68.1	20.0	12.0
100:0	5.0	-	61.0	70.3	14.8	14.9	68.9	73.7	13.8	12.5
100:0	-	5.0	59.2	68.0	16.8	15.3	594	75.2	12.0	12.9
100:0	2.5	2.5	60.0	68.8	15.9	15.4	59.8	77.4	9.6	13.1
75:25	-	-	46.7	62.1	27.6	10.4	75.6	67.3	17.4	15.4
75:25	5.0	-	63.1	64.5	14.8	20.7	76.9	69.1	14.5	16.5
75:25	-	5.0	67.6	65.6	15.0	19.5	70.7	71.1	11.9	17.0
75:25	2.5	2.5	63.4	64.7	14.9	20.5	68.8	69.9	12.8	17.4
50:50	-	-	65.3	59.6	18.4	22.1	80.2	60.7	18.4	20.9
50:50	5.0	-	62.0	64.6	15.6	19.9	71.7	62.1	16.5	21.5
50:50	-	5.0	67.5	60.6	17.7	21.8	74.9	60.8	19.0	20.3
50:50	2.5	2.5	63.4	58.3	18.3	23.4	80.1	60.7	17.5	21.8
25:75	-	-	69.7	55.6	21.8	22.8	68.9	52.2	21.6	26.2
25:75	5.0	-	73.5	55.2	23.8	21.1	72.6	56.3	16.2	27.6
25:75	-	5.0	65.9	57.5	21.0	21.5	70.2	54.3	18.1	27.7
25:75	2.5	2.5	66.6	60.0	20.3	19.8	76.9	53.3	20.4	26.4
SEM			4.36	2.63	3.35	2.13	5.27	1.73	1.89	0.60
Effect of R:C ratio										
100:0	-	-	59.3^{b}	71.3^{a}	14.3^{b}	14.4^{c}	65.0^{b}	73.6^{a}	13.9^{b}	12.6^{d}
75:25	-	-	60.2^{b}	64.2^{b}	18.0^{ab}	17.7^{b}	73.0^{ab}	69.4^{b}	14.1^{b}	16.5^{c}
50:50	-	-	65.2^{ab}	60.8^{bc}	17.5^{ab}	21.8^{a}	72.1^{ab}	61.1^{c}	17.8^{a}	21.1^{b}
25:75	-	-	68.9^{a}	57.1^{c}	21.7^{a}	21.3^{a}	76.7^{a}	54.0^{d}	19.1^{a}	27.0^{a}
Linear	-	-	**	**	*	**	*	**	**	**
Quadratic	-	-	ns	ns	ns	ns	*	ns	ns	**
Cubic	-	-	ns	ns	ns	ns	ns	ns	ns	nss
Effect of oil supplementation										
Un-supplement	-	-	59.7	63.9	19.4	16.8	74.1	62.1^{b}	19.3^{a}	18.6^{b}
CO	-	-	64.9	63.6	17.2	19.1	72.5	65.3^{a}	15.2^{b}	19.5^{ab}
WO	-	-	65.1	62.9	17.6	19.5	68.8	65.3^{a}	15.2^{b}	19.5^{ab}
CW	-	-	64.0	62.9	17.3	19.7	71.4	65.3^{a}	15.1^{b}	19.7^{a}
RC*Oil interaction	-	-	ns	ns	ns	ns	ns	ns	ns	ns
Treatment[1]			72 h				96 h			
100:0	-	-	84.5	69.4	18.7	12.0	105.6	60.5	19.4	20.1
100:0	5.0	-	73.1	73.0	14.1	12.9	101.0	64.7	20.2	15.1
100:0	-	5.0	52.8	68.2	18.7	13.2	120.8	64.1	19.2	16.7
100:0	2.5	2.5	86.8	73.1	14.5	12.5	111.1	67.1	14.6	18.4
75:25	-	-	85.5	65.4	18.9	15.8	122.7	62.5	18.2	19.4
75:25	5.0	-	78.3	70.1	12.9	17.1	115.2	61.3	18.2	20.6
75:25	-	5.0	68.1	69.0	14.3	16.8	100.2	58.5	17.4	24.2
75:25	2.5	2.5	84.3	70.4	12.9	16.8	103.7	61.5	17.7	20.9
50:50	-	-	89.3	59.8	19.4	20.8	111.5	60.5	18.5	21.0
50:50	5.0	-	75.0	618	17.2	21.0	97.4	58.8	17.6	23.7
50:50	-	5.0	72.4	58.0	20.3	21.8	99.2	59.5	17.0	23.5
50:50	2.5	2.5	72.0	58.3	18.1	23.7	103.3	61.6	17.8	20.7
25:75	-	-	95.7	54.3	20.4	25.4	101.1	62.7	19.7	17.6
25:75	5.0	-	88.4	54.3	19.1	26.7	98.3	60.4	20.1	19.6
25:75	-	5.0	71.9	55.3	15.3	29.5	100.1	61.9	18.8	19.4
25:75	2.5	2.5	89.4	54.6	18.0	27.5	102.7	61.5	19.1	19.5
SEM			6.05	1.06	1.29	0.58	4.82	0.69	0.63	0.77
Effect of R:C ratio										
100:0	-	-	74.4^{b}	70.9^{a}	16.5^{bc}	12.6^{d}	109.6^{ab}	64.1^{a}	18.4^{b}	17.5^{c}
75:25	-	-	77.1^{ab}	68.7^{b}	14.7^{c}	16.6^{c}	110.4^{a}	61.6^{b}	17.9^{b}	21.2^{a}
50:50	-	-	79.0^{ab}	59.5^{c}	18.7^{a}	21.8^{b}	102.8^{bc}	60.9^{bc}	17.7^{b}	22.2^{a}
25:75	-	-	86.3^{a}	54.6^{d}	18.2^{ab}	27.2^{a}	100.5^{c}	60.1^{c}	19.4^{a}	19.0^{b}
Linear	-	-	*	**	**	**	**	**	*	**
Quadratic	-	-	ns	*	ns	*	ns	**	**	**
Cubic	-	-	ns	**	**	ns	ns	ns	ns	ns
Effect of Oil supplementation										
Un-supplement	-	-	88.9^{a}	62.2^{c}	19.3^{a}	18.5^{b}	110.2	61.5^{b}	19.0^{a}	19.5^{b}
CO	-	-	78.7^{b}	64.8^{a}	15.8^{b}	19.4^{a}	103.0	61.3^{b}	19.0^{a}	19.7^{b}
WO	-	-	72.3^{c}	62.6^{bc}	17.1^{b}	20.3^{a}	105.1	61.0^{b}	18.1^{ab}	21.0^{a}
CW	-	-	83.1^{ab}	64.1^{ab}	15.8^{b}	20.1^{a}	105.2	62.9^{a}	17.3^{b}	19.8^{ab}
RC*Oil interaction	-	-	ns	*	ns	*	*	**	**	**

[1] R:C, roughage to concentrate ratio; CO, coconut oil (5.0% of substrate)
WO, wild almond seed oil (5.0% of substrate); CW, 2.5% CO and 2.5% WO mix
[2] TVFA, total volatile fatty acid (mmol/L); C2, acetic acid (% of TVFA)
C3, propionic acid (% of TVFA); C4, butyric acid (% of TVFA)
[a-d] Values on the same column and factor with different superscripts differed ($p<0.05$)
**$p<0.01$; *$p<0.05$; ns, non-significantly different; SEM, standard error of the mean

Table 3: Effect of R:C ratio and oil supplementation on calculated methane production

Treatment[1]			Methane, mol/100 mol VFA				Methane, mmol/g substrate			
R:C	CO	WO	24 h	48 h	72 h	96 h	24 h	48 h	72 h	96 h
100:0	-	-	37.3	29.9	30.9	29.9	105.2	107.2	131.0	158.1
100:0	5.0	-	33.5	34.3	34.2	29.6	102.2	116.7	125.1	149.4
100:0	-	5.0	32.1	35.7	30.8	30.2	95.0	106.0	81.4	182.6
100:0	2.5	2.5	32.7	37.4	33.9	33.5	98.1	111.6	146.7	186.1
75:25	-	-	24.5	31.7	30.5	30.8	62.7	119.7	130.6	189.0
75:25	5.0	-	33.2	33.7	34.8	30.8	104.7	129.5	136.2	177.3
75:25	-	5.0	33.2	35.5	33.8	31.2	112.2	124.8	114.8	156.2
75:25	2.5	2.5	33.2	34.9	34.9	31.1	105.2	120.0	146.7	161.4
50:50	-	-	30.6	30.6	29.9	30.5	99.7	122.7	133.2	170.3
50:50	5.0	-	32.7	32.0	31.5	31.1	101.4	114.5	117.5	151.5
50:50	-	5.0	31.1	30.3	29.2	31.5	104.8	113.3	105.8	156.1
50:50	2.5	2.5	30.6	31.2	30.7	31.1	101.0	124.4	110.4	160.7
25:75	-	-	28.1	28.0	28.9	29.8	97.8	96.5	138.5	150.5
25:75	5.0	-	26.7	31.9	29.8	29.5	98.2	115.5	131.6	144.7
25:75	-	5.0	28.7	30.5	32.5	30.4	94.4	106.9	116.7	152.4
25:75	2.5	2.5	29.3	28.9	30.6	30.2	97.0	111.1	136.7	155.0
SEM	2.38	1.37	0.92	0.44	9.15	7.23	9.23	7.56		
Effect of R:C ratio										
100:0	-	-	33.9[a]	34.3[a]	32.4[a]	30.8[a]	100.1	110.4[b]	121.1ab	169.1[a]
75:25	-	-	31.0[ab]	33.9[a]	33.5[a]	31.0[a]	96.2	123.5[a]	132.1[a]	171.0[a]
50:50	-	-	31.2[ab]	31.0[b]	30.3[b]	31.1[a]	101.7	118.7[ab]	116.7[b]	159.6[ab]
25:75	-	-	28.2[b]	29.8[b]	30.5[b]	30.0[b]	96.9	107.5[b]	130.9[ab]	150.6[b]
Linear	-	-	**	**	**	*	ns	ns	ns	**
Quadratic	-	-	ns	ns	ns	*	ns	**	ns	ns
Cubic	-	-	ns	ns	**	ns	ns	ns	*	ns
Effect of Oil supplementation										
Un-supplement	-	-	30.1	30.0[b]	30.1[b]	30.3[b]	91.4	111.5	133.4[a]	167.0
CO	-	-	31.6	33.0[a]	32.6[a]	30.2[b]	101.6	119.1	127.6[a]	155.7
WO	-	-	31.3	33.0[a]	31.6[a]	30.8[ab]	101.6	112.7	104.7[b]	161.8
CW	-	-	31.5	33.1[a]	32.5[a]	31.5[a]	100.3	116.8	135.1[a]	165.8
RC*Oil interaction	-	-	ns	ns	ns	**	ns	ns	ns	*

[1] R:C, roughage to concentrate ratio; CO, coconut oil (5.0% of substrate)
WO, wild almond seed oil (5.0% of substrate); CW, 2.5% CO and 2.5% WO mix
Methane (CH_4) = 0.45 acetate -0.275 propionate + 0.40 butyrate (Moss *et al*., 2000)
[a-b] Values on the same column and factor with different superscripts differed ($p<0.05$)
**$p<0.01$; *$p<0.05$; ns, non-significantly different; SEM, standard error of the mean

respectively); in contrast, only +0.25 of hydrogen from butyric acid is presented in the equation of Orskov *et al.* (1968). Therefore, estimation of methane production by using the equation based on volatile fatty acid proportion must be closely considered. Wild almond seed oil supplementation showed lowest methane production (mmol/g substrate) at 72 h of incubation. This result agreed with Panyakaew *et al.* (2008) who also found MCFA from coconut oil and wild almond oil did reduce the rumen methane production. However, the reasons of opposite effect of MCFA from wild almond seed oil as compared to coconut oil are still unclear.

Conclusion: It could be concluded that increasing level of concentrate proportion increased while plant oil supplementation decreased *in vitro* fermentation end-products. Volatile fatty acid production was affected by R:C ratio and oil supplementation interaction in late of incubation. Utilization of wild almond seed oil as feed supplement for ruminant feeding should be further conducted in the production trial.

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