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Effect of rumen-protected fat on *in vitro* rumen fermentation and apparent biohydrogenation of fatty acids

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ABSTRACT

This study aimed to evaluate the effects of rumen-protected fat (RPF) on *in vitro* fermentation profiles and biohydrogenation of fatty acids. The treatment diets were basal diet (70:30 concentrate to rice straw) with no RPF (CON), basal diet plus prilled fat (PF), basal diet plus prilled fat with lecithin (PFL) and basal diet plus calcium soap of palm fatty acids (CaS). *In vitro* gas production, fermentation kinetics, *in vitro* dry matter digestibility (IVDMD), *in vitro* organic matter digestibility (IVOMD), metabolizable energy (ME), rumen fermentation and fatty acid profile were determined. The results show that RPF did not affect cumulative gas production and gas production kinetics. PFL significantly ($p < 0.05$) improved IVDMD and IVOMD, although the addition of RPF did not affect ME. The volatile fatty acid (VFA), pH, ammonia nitrogen, methane, and molar proportion of VFA were not significantly influenced by the RPF; methane was numerically reduced because of PFL treatment. The concentration of monounsaturated and polyunsaturated fatty acids increased ($p < 0.05$) whereas that of saturated fatty acids decreased in the control diet. The biohydrogenation of C18:2n-6 and C18 unsaturated fatty acids was enhanced ($p < 0.05$) by PFL. These findings suggest that PFL enhances gas production, decreases methane and increases the biohydrogenation of C18:2n6 without disrupting rumen fermentation.

Keywords: *Biohydrogenation; Fatty acid; Gas production; In vitro fermentation; Rumen-protected fat*

INTRODUCTION

Dietary fats are used in ruminant nutrition to increase ruminant diets' energy density. Nevertheless, high concentrations of dietary fats could influence rumen metabolism adversely by reducing rumen pH causing rumen acidosis (Hartati *et al*., 2012), and extensive hydrolysis in the rumen resulting in reduced fibre digestibility, which can affect subsequent production performance in

livestock (Szumacher-Strabel *et al*., 2009). These adverse effects of high levels of dietary fats can be shunned by protecting fats from hydrolysis in the rumen by rumen microbes thus making them rumen-protected fats (RPF) or rumen inert or rumen bypass fats. Hence, RPF can improve the energy density of the diet without affecting rumen fermentation.

The *in vitro* gas production technique is extensively used to evaluate the nutritional values of different ruminant feedstuffs, and to predict their fermentation, digestibility and metabolizable energy contents (Deutschmann *et al*., 2017). The rumen-protected fats in the form of potassium soaps depressed gas production, *in vitro* true digestibility, total VFA production, and ammonia nitrogen concentration (Getachew *et al*., 2001). The addition of calcium soap-flaxseed oil *in vitro* significantly increased total VFA production and supplementation of micro-encapsulated flaxseed oil produced the highest propionate concentration and H_2 utilization, the lowest A:P ratio and methane production, and did not disturb rumen microbial activity (Hidayah *et al.*, 2014). Wettstein *et al.* (2001) reported that there was a significant influence of partial replacement of commercially available RPF by lecithin on the utilization and digestion of fatty acids.

Many studies on different types of protected fat for *in vitro* fermentation have been conducted (Álvarez-Torres *et al*., 2024). However, prilled fat with lecithin and calcium soaps were not previously studied for *in vitro* rumen fermentation, *in vitro* dry matter digestibility, *in vitro* organic matter digestibility and methane production. Therefore, the present research was conducted to evaluate the effects of RPF in the form of prilled fat, prilled fat with lecithin and calcium soaps of palm fatty acids on *in vitro* rumen fermentation and kinetics; gas production, volatile fatty acid profile and apparent biohydrogenation of fatty acids.

MATERIALS AND METHODS

Animals, Substrates, and Treatments

Three male Dorper sheep weighing 25.17 ± 0.44 kg with permanent rumen fistula were used as rumen liquor donors. A completely randomized design was used with three replications in three incubation runs. The rumen-protected fat (RPF) (Table 1) was obtained from two different companies. The substrates were four experimental diets (1) basal diet (rice straw, corn starch, soybean meal, palm oil, calcium carbonate, sodium chloride, vitamin-mineral mix) with no added RPF (CON); (2) basal diet plus prilled fat (PF); (3) basal diet plus prilled fat with lecithin (PFL); and (4) basal diet plus calcium soap (calcium salts of palm fatty acids) (CaS) on dry matter basis at 5 % of the DM of the substrates Table 2.

Chemical Analysis

Proximate analysis of the diets was determined according to the protocol of AOAC (2007) and Van Soest *et al*. (1991).

Table 1. Fatty Acid Composition of Rumen-Protected Fats

Fatty acids (% of total FA)	PF	PFL	CaS
C15:0	1.39	1.15	1.43
C16:0	72.98	76.72	48.31
C16:1n-9	0.16	0.05	0.81
C18:0	5.16	4.92	4.33
C18:1n-9	16.34	12.85	41.15
C18:2n-6	3.40	3.94	1.64
C18:3n-3	0.57	0.37	2.33
ΣSFA [1]	79.53	82.79	54.07
ΣMUFA	16.5	12.90	41.95
ΣPUFA	3.97	4.31	3.97
n-6: n-3	5.96	10.65	0.70

PF = Prilled fat, PFL = Prilled fat with lecithin, CaS = Calcium soap of palm fatty acids

[1] Calculated ΣSFA = Total saturated fatty acid (C15:0+C16:0+C18:0), ΣMUFA = Total monounsaturated fatty acid (C16:1+C18:1), ΣPUFA = Total polyunsaturated fatty acid (C18:2n-6+C18:3n-3) n-6: n-3 = (C18:2n-6÷C18:3n-3).

Table 2. Ingredients, Chemical and Fatty Acid Composition of Experimental Diets

Ingredients (%)	Diets				SEM	P value
	CON	PF	PFL	CaS		
Rice straw (urea treated)	29	33	34	35	--	--
Corn starch	39	31	30	29	--	--
Soybean meal	26	27	27	27	--	--
Palm oil	4	2	2	2	--	--
Calcium carbonate	1	1	1	1	--	--
Sodium chloride	0.5	0.5	0.5	0.5	--	--
Vitamin-mineral mix [1]	0.5	0.5	0.5	0.5	--	--
PF	--	5	--	--	--	--
PFL	--	--	5	--	--	--
CaS	--	--	--	5	--	--
Total	100	100	100	100	--	--
Chemical composition (% DM)						
Dry matter	91.54	92.08	92.31	91.95	0.176	NS
Organic matter	93.29	91.23	91.31	91.96	0.346	NS
Ether extract	4.98	8.27	8.04	3.38	0.599	*
Crude protein	18.88	18.57	19.92	19.23	0.621	NS
Crude fibre	12.67	28.42	30.09	13.77	2.500	*
Neutral detergent fibre	56.06	58.78	60.22	51.32	1.209	*
Acid detergent fibre	17.89	21.80	24.33	23.94	0.585	NS
Acid detergent lignin	6.65	13.33	10.35	4.92	1.173	*
Metabolizable energy (MJ/kg DM)	11.68	11.65	11.66	11.68	0.274	NS
Fatty acids (% of total FA)						
C15:0	0.78	1.12	0.95	0.60	0.058	*
C16:0	32.93	59.52	61.49	21.21	5.201	NS
C16:1n-9	0.20	0.13	0.11	0.30	0.023	*
C18:0	3.78	4.87	4.58	2.62	0.264	NS
C18:1n-9	42.38	24.20	21.83	52.70	3.867	*
C18:2n-6	18.93	9.69	10.44	21.51	1.560	*
C18:3n-3	1.00	0.47	0.60	1.06	0.077	NS
ΣSFA [2]	37.49	65.51	67.03	24.43	5.508	*
ΣMUFA	42.58	24.33	21.94	53.00	3.890	*
ΣPUFA	19.93	10.16	11.04	22.56	1.636	*
n-6: n-3	18.93	20.62	17.40	20.29	0.445	*

RPF = Rumen protected fat, CON = Basal diet without RPF, PF = Basal diet + prilled fat, PFL = Basal diet + prilled fat with lecithin, CaS = Basal diet + calcium soap, NS = Non-significant, '*'= significantly different at ($P<0.05$), [1] Contained (g/kg) $CuSO_4.5H_2O$, 70; $ZnSO_4.7H_2O$, 240; $FeSO_4.7H_2O$, 170; $MnSO_4.5H_2O$, 290; (mg/kg) $CoCl2.6H_2O$ 510; KI, 220; Na_2SeO, 130; vitamin B1, 450; pantothenic acid, 750; vitamin K_3, 150; folic acid, 15; vitamin B_{12}, 0.9; vitamin B_5, 1,050; (IU), vitamin D_3, 324,000; vitamin A, 620,000. [2] calculated. ΣSFA = Total saturated fatty acid (C15:0+C16:0+C18:0), ΣMUFA = Total monounsaturated fatty acid (C16:1+C18:1), ΣPUFA = Total polyunsaturated fatty acid (C18:2n-6+C18:3n-3) n-6: n-3 = (C18:2n-6÷C18:3n-3).

Collection of Rumen Liquor

The *in vitro* experiment was conducted using the procedure of Menke and Steingass (1988).The rumen liquor was obtained from the rumen-fistulated animals before morning feeding into a pre-warmed thermos flask, continuously flushed with CO_2 and immediately transported to the laboratory. The rumen liquor was pooled together and filtered through six layers of cheesecloth.

Preparation of Buffered Media

The buffered media was prepared by mixing

five different solutions consisting of Solution A (micromineral), Solution B (buffer), Solution C (macro mineral), Resazurin and reducing solution. The strained rumen liquor was added to the media in a ratio of 1:2 (v/v). The mixture was kept stirred and then placed in a water bath at 39 °C under constant CO_2 flushing.

***In Vitro* Rumen Fermentation of Samples**

Each sample (200 mg) was placed in a 100 mL calibrated glass syringe fitted with a rubber tube and about 30 ml rumen liquor-buffer medium was added. A pre-lubricated piston was inserted into the syringe and pressed forward to remove air from the syringe through the rubber tube. The rubber tube was sealed with a plastic clip and the initial gas volume was read at the point where the end mark of the piston lies. The *in vitro* gas production was measured by incubating the samples for 72 hours at 39°C in a water bath and the gas produced was recorded at 0, 3, 6, 9, 12, 24, 36, 48 and 72 hours of incubation. The pH of the rumen fluid was taken with a Mettler-Toledo pH meter (Mettler-Toledo, Ltd England).

Gas Production Kinetics

The cumulative gas production data was fitted to the model $y = a + b\ (1 - e^{-ct})$ (Orskov and Mcdonald, 1979) by using the NEWAY Excel software.

***In Vitro* Dry Matter and Organic Matter Digestibility**

After 72 hours of incubation, the fermentation residues were shifted into pre-weighed and pre-dried beakers. The beakers along with the contents were placed into an oven at 105°C for 24 h to dry and *in vitro* dry matter digestibility (IVDMD) was calculated. The dried contents were ignited in a furnace at 550°C to measure organic matter digestibility (IVOMD). The following equation was used:

IVDMD or IVOMD (%):

{[Initial DM or OM (g) – Undigested DM or OM (g) – Blank] / Initial DM or OM (g)} X 100%

Determination of Volatile Fatty Acids

Volatile fatty acids (VFA) were analysed after 72 hours, the rumen liquor was fixed by adding 25% metaphosphoric acid ratio 4:1 (v/v). The mixture was centrifuged at 3000 g for 10 min and the supernatant (0.5 ml) was collected and mixed with 0.5 ml 20 mM methyl n-valeric acid (internal standard) and was analysed by gas chromatograph (Hewlett Packard 6890 GC system) with bonded phase fused silica capillary column (15m, 0.32mm ID, 0.25 µm film thickness) equipped with a flame ionization detector (FID).

Determination of Ammonia Nitrogen

Ammonia nitrogen (NH_3-N) was determined using the colorimetric method described by Soloranzo (1969). A standard curve was made to know the linear relationship between the varying concentrations of ammonium sulphate standard solution and the intensity of colour produced. This intensity was measured at a wavelength of 420 nm by a spectrophotometer (Secomam, Domont, France) within 5–10 minutes after placing it at 0 absorbance with the blank.

Estimation of Methane and Metabolizable Energy

Methane was estimated using the equation proposed by Widiawati and Thalib (2007). Metabolizable energy (ME) was estimated by using the protocol of Menke and Steingass (1988).

Fatty Acid Analysis

Total FA from each substrate (1.0 g) and rumen liquor (10 mL) was extracted in chloroform: methanol (2:1, v/v) mixture according to Rajion *et al*. (1985).

Biohydrogenation of Fatty Acids

The rate of disappearance or apparent biohydrogenation of C18:3n-3, C18:2n-6 and C18:1n-9 was calculated by using the equation of Vlaeminck *et al*. (2008):

Apparent biohydrogenation (%):

$100 \text{ X } [(CFA)_i - (CFA)_f] / (CFA)_i$

in which (CFA)i = % concentration of unsaturated FA at 0-hour incubation, $(CFA)_f$ = % concentration of unsaturated FA at 72 hours of incubation.

Statistical Analysis

The data obtained were analysed using one-

way analysis of variance (ANOVA) by a general linear model (GLM) procedure in SAS software 9.4 Version (SAS Institute Inc., Cary, NC, USA). Duncan's multiple range test was used to separate means at $p < 0.05$ significance level.

RESULTS

In Vitro Gas Production

In vitro gas production and rate of gas production per hour did not differ significantly. When the incubation time was extended from 0 to 72 hours, the cumulative gas production increased linearly (Figure 1). The first 24 hours of incubation produced maximum gas or almost half of the total gas volume; the second 24 hours produced the least gas, and the final 24 hours produced the least gas.

In Vitro Gas Production Kinetics

The gas production kinetics and net gas production (NGP) were not significantly different between the treatments. Overall, the diet without added RPF produced the most amount of gas, followed by the PFL diet and CaS, while the PF produced the least amount of gas (Table 3).

The digestibility values of IVDMD and IVOMD showed a significant difference ($P<0.05$), with the highest values found in PFL and the lowest values in CaS. Additionally, Table 3 shows that there was no significant difference in ME between the treatments.

The parameters estimated from gas production kinetics, 'a' (gas produced from the soluble fraction), 'b' (gas produced from the insoluble fraction and 'c' (rate of gas production) and potential gas production (a+b) did not differ significantly across the treatments (Table 3).

In Vitro Fermentation Characteristics

The *in vitro* rumen fermentation parameters, such as pH, CH_4, and NH_3-N, did not exhibit a significant difference between the treatments. RPF supplementation did not substantially influence the concentration of total VFA or the molar proportions of acetate, propionate, isobutyrate, butyrate, or the ratio of acetate to propionate (Table 4).

Fatty Acid Composition of Rumen Liquor and Apparent Biohydrogenation

There was no discernible difference in the quantities of C12:0, C14:0, C15:0, C15:1, C16:0, C16:1n-7, and *cis*-7. Among the treatments were C16:1n-9, C18:0, C18:1n-9, C18:2n-6, C18:3n-3, CLA *cis*-9 *trans*-11, and CLA *trans*-10 *cis*-12. The concentration of C18:1 *trans* 11 varies significantly ($P<0.05$), with the highest concentra-

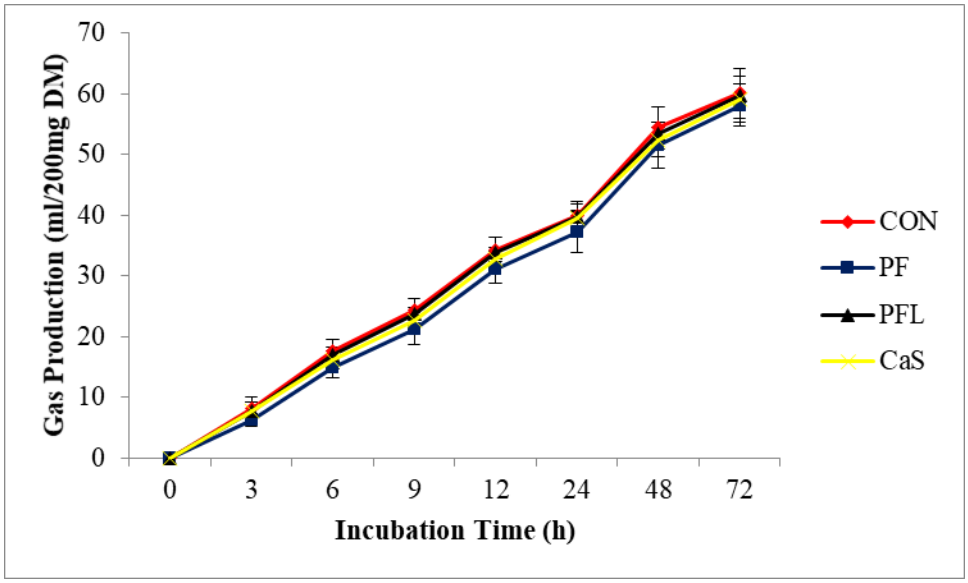

Figure 1. *In vitro* cumulative gas production profile. The incubation time was from 0 to 72 hours. The total gas volume produced was measured in ml per 200 mg DM. Data points are mean values based on triplicate determination. Error bars represent the standard error of the mean.

Table 3. In Vitro Gas Production, Gas Production Kinetics and Digestibility with Different Rumen-Protected Fats

Parameters	Treatments					
	CON	PF	PFL	CaS	SEM	*P-value*
3H	8.17	6.25	7.83	7.67	0.332	0.183
6H	17.67 [a]	14.83 [b]	17.08 [a]	16.13[ab]	0.371	0.027
9H	24.33 [a]	21.06 [b]	23.83 [a]	22.75[ab]	0.419	0.026
12H	34.43 [a]	31.17 [b]	33.83 [a]	32.75[ab]	0.419	0.026
24H	39.92	37.25	39.75	39.50	0.520	0.235
48H	54.42	51.50	53.42	52.50	0.615	0.398
72H	60.19	58.07	59.67	59.23	0.671	0.774
Rate (mL/h)	0.84	0.81	0.83	0.82	0.009	0.756
NGP (mL)	60.19	58.07	59.67	59.23	0.671	0.774
IVDMD (%)	72.66 [a]	68.34 [b]	73.92 [a]	67.88 [b]	0.641	<0.0001
IVOMD (%)	63.82 [a]	59.97 [b]	64.45 [a]	61.08 [b]	0.561	0.004
ME (MJ/kg DM)	8.74	8.37	8.79	8.71	0.073	0.157
Degradation (Constant from the fitted model)						
a (mL)	20.23	20.48	20.00	19.61	0.268	0.723
b (mL)	76.44	74.35	75.93	75.28	0.708	0.773
c (mL h^{-1})	0.058 [a]	0.052 [b]	0.058[ab]	0.056[ab]	0.001	0.105
a + b (mL)	96.67	94.84	95.93	94.89	0.653	0.737

'a'= volume of gas produced from soluble fraction; 'b' = volume of gas produced from insoluble fraction; 'c' = gas production rate constant from the insoluble fraction; 'a+b' = potential degradability, NGP=net gas production, IVDMD= in vitro dry matter digestibility; IVOMD= in vitro organic matter digestibility; [a,b,c], means with a different superscript in each row differ significantly (P<0.05); SEM=Standard error of means; CON = Basal diet without RPF, PF = Basal diet + prilled fat, PFL = Basal diet + prilled fat with lecithin, CaS = Basal diet + calcium soap.

Table 4. In Vitro Rumen Fermentation Characteristics of Substrates with Different Rumen-Protected Fats

Parameters	Treatments					
	CON	PF	PFL	CaS	SEM	*P-value*
pH	6.66	6.74	6.70	6.69	0.014	0.261
CH_4 (mM/L)	7.72	7.14	6.88	7.14	0.185	0.453
NH_3-N (mg/dL)	15.46 [a]	12.30 [b]	15.33 [a]	13.49 [ab]	0.528	0.091
Total VFA (mM)	33.23	30.41	30.08	31.05	1.052	0.741
Molar proportion (%)						
Acetate (A)	43.06	44.15	43.44	43.58	0.193	0.252
Propionate (P)	28.84	28.80	29.39	29.20	0.334	0.922
Isobutyrate	9.94	9.64	9.73	9.84	0.112	0.812
Butyrate	18.16	17.41	17.43	17.38	0.453	0.925
A:P	1.50	1.54	1.48	1.50	0.017	0.737

[a,b,c], means with different superscripts in each row differ significantly (P<0.05); Standard error of means; CON = Basal die without RPF, PF = Basal diet + prilled fat, PFL = Basal diet + prilled fat with lecithin, CaS = Basal diet + calcium soap.

tion found in CON and the lowest in PF. In PF, the highest concentrations were in C12:0, C15:0, C16:0, and C16:1n-9, whereas in PFL, the lowest concentrations were found in C12:0, C14:0, C15:1, C18:2n-6, and CLA cis-9 trans-11. As shown in Table 5, CLA trans-10 cis-12 was the highest in PFL and lowest in CON, whereas CLA trans-9 trans-11 was the highest in PF and the lowest in CON. The total Saturated fatty acid (SFA) content was significantly different (P<0.05) between the treatments, with PFL was the largest proportion and CON was the lowest. Furthermore, there is a significant difference (P<0.05) in the levels of monounsaturated fatty acids (MUFA) between the treatments, but not in the polyunsaturated fatty acids (PUFA). Both,

Table 5. Fatty Acid Composition of Rumen Liquor at 72 Hours of Incubation of Substrates with Different Rumen-Protected Fats

Fatty acids (% of total FA)	Treatments					
	CON	PF	PFL	CaS	SEM	*P-value*
C12:0	1.27	1.36	0.98	1.34	0.114	0.655
C14:0	3.45	3.20	2.85	3.20	0.115	0.340
C15:0	1.17	1.29	1.20	1.29	0.059	0.873
C15:1	3.01	3.06	2.82	3.37	0.118	0.453
C16:0	23.26	26.41	26.24	23.30	0.598	0.079
C16:1n-7	1.58	1.57	1.50	1.46	0.063	0.913
C16:1n-9	1.87	1.95	1.73	1.61	0.092	0.616
C18:0	50.60	50.37	52.52	52.48	0.854	0.731
C18:1 trans 11	6.84 [a]	5.08 [b]	5.28 [b]	6.29 [ab]	0.262	0.044
C18:1n-9	2.92	2.21	2.03	1.94	0.261	0.563
C18:2n-6	1.78	1.39	1.04	2.03	0.185	0.248
C18:3n-3	0.96	0.87	0.64	0.51	0.093	0.316
CLA cis-9 trans-11	0.72	0.67	0.46	0.64	0.051	0.307
CLA trans-10 cis-12	0.54	0.58	0.70	0.59	0.076	0.903
n-6 PUFA	1.78	1.39	1.04	2.03	0.185	0.248
n-3 PUFA	0.96	0.87	0.64	0.51	0.093	0.316
C18 UFA	13.76 [a]	10.79 [b]	10.15 [b]	12.00 [ab]	0.486	0.034
∑SFA	79.78 [b]	82.63 [a]	83.80 [a]	81.56 [ab]	0.508	0.025
∑MUFA	16.22 [a]	13.87 [b]	13.37 [b]	14.67 [ab]	0.378	0.030
∑PUFA	2.74	2.26	1.68	2.55	0.202	0.278
∑UFA	20.22 [a]	17.37 [b]	16.20 [b]	18.44 [ab]	0.508	0.025
n6: n3	3.73	1.88	2.68	6.41	0.801	0.210
UFA: SFA	0.25 [a]	0.21 [b]	0.19 [b]	0.23 [ab]	0.008	0.022
PUFA: SFA	0.03	0.03	0.02	0.03	0.003	0.130
MUFA: SFA	0.20 [a]	0.17 [b]	0.16 [b]	0.18 [ab]	0.006	0.019

[a, b, c,] means having different superscripts in each row are significantly different (P<0.05). Standard error of means, CON = Basal diet without RPF, PF = Basal diet + prilled fat, PFL = Basal diet + prilled fat with lecithin CaS = Basal diet + calcium soap, ∑SFA = total saturated fatty acids, ∑UFA = total unsaturated fatty acids ∑MUFA = total monounsaturated fatty acids, ∑PUFA = total polyunsaturated fatty acids.

however, were discovered to be lowest in PFL and higher in the CON diet (Table 5).

Following a 72-hour incubation period, the apparent biohydrogenation of C18:1n-9, C18:2n-6, C18:3n-3, and C18 UFA did not differ substantially across the treatments (Table 6). C18:1n -9 and C18 UFA levels were found to be lower in CON and higher in PF. While C18:3n-3 concentrations were found to be higher in CaS and lower in CON, the rate of biohydrogenation of C18:2n-6 was observed to be higher in PFL and lower in CaS.

DISCUSSION

The highest amount of gas produced from the diet without added RPF could be due to differences in the chemical composition of diets as the diet without RPF had low ash and low crude fibre contents compared with other treatment groups. The lowest amount of gas produced from PF could also be due to high ash and crude fibre contents as *in vitro* gas production is affected by the changes in the chemical composition of the diets (Kilic and Garipoglu, 2009), proportion of feed ingredients (Maccarana *et al.*, 2016), and amount of ash in the diets (high ash leads to low gas production) (Menke and Steingass, 1988).

The high cumulative gas production in the present experiment could be attributed to high

Table 6. Apparent Biohydrogenation of Fatty Acids at 72 Hours of Incubation of Substrates with Different Rumen-Protected Fats

	Treatments					
Apparent Biohydrogenation (%)	CON	PF	PFL	CaS	SEM	*P-value*
18:1n-9	67.74	71.84	69.17	70.01	1.001	0.561
18:2 n-6	74.85	78.51	80.14	72.87	1.696	0.434
18:3 n-3	76.34	78.64	84.85	86.18	2.066	0.274
C18 UFA	38.57	46.52	46.46	45.99	1.841	0.360

SEM= Standard error of means, CON = Basal diet without RPF, PF = Basal diet + Prilled fat, PFL = Basal diet + Prilled fat with lecithin, CaS = Basal diet + calcium soap.

concentrate diets (about 70%), similar to the results reported by Kang and Wanapat (2013). Concentrates produce significantly higher levels of gas as compared to straws and forages because concentrates are rich in non-structural carbohydrates which are readily fermented by rumen microbes yielding more gas. Though gas production kinetics was not significantly between the treatments, yet it was numerically higher in the diet without RPF, which could be because of the same concentrate diet used throughout all treatment groups; secondly, RPF in diets was inert in the incubation medium and hence did not affect the microbial activity of fibre digestion ultimately resulting in the non-significant difference in gas production kinetics.

The NDF and ADF concentration of forages can also affect their fermentation. An increase in NDF and ADF decreases gas production during incubation and vice versa. This finding is also supported by Maccarana *et al.* (2016), thus, the low concentration of NDF and ADF in the diet without RPF in the present study could have resulted in increased gas production.

The IVDMD and IVOMD were highly significantly different among the treatments being the highest in PFL because of the presence of lecithin along with RPF. A study conducted by Wettstein *et al.* (2001) found noticeable effects of partial replacement of commercially available RPF by lecithin on utilization and digestion of fatty acids (FA). This effect of lecithin could be because of their ability to disperse in water and they have a gradually disappearing portion. Thus, after lecithin are consumed, attachment to feed particles or rumen microbes could be less prominent and the release of the FA could be slowed down ultimately causing less adverse influence on rumen fermentation (Nagaraja *et al.*, 1997). Similarly, Hartati *et al.* (2012) found a significant difference in IVDMD with RPF-protein supplement formulations in the rumen and post-rumen digestion. Contrary, Naik *et al.* (2009) found a non-significant influence of RPF on IVDMD.

No significant difference in *in vitro* rumen pH among the treatments was expected as the gas production parameters, molar concentration and individual VFA were similar across the treatments. The chemical composition of the diets also did not affect rumen pH as gas production was influenced by differences in NDF and ADF concentrations in the present study. Similarly, Maccarana *et al.* (2016) found that changing the chemical composition of diets did not influence rumen pH. There was numerically a slight decrease in ruminal pH in the diet without RPF which could be associated with the addition of palm oil because dietary oils possess pH reducing the effect, in a study conducted by Hidayah *et al.* (2014) reported that supplementation of non-protected vegetable oil significantly decreased rumen pH. Moreover, an increase in propionate and butyrate production could reduce pH values as a study conducted by Günal *et al.* (2017) reported a reduction in pH values associated with numerical increases in propionate and butyrate. Another reason could be that a high-concentrate diet is known to lower rumen pH and lead to depressing fibre degradability due to modification of the rumen microbial population.

PFL decreased CH_4 production numerically without decreasing total gas production and rumen fermentation. Similar findings were reported by Wettstein *et al.* (2000). Minimum methane reduction in the current study was obtained from the diet containing palm oil without RPF (CON). Though there is reported evidence of methane-reducing effects of dietary lipids (Vargas *et al.*, 2017), there was very little methane reduction

from the diet containing palm oil without RPF, possibly other three diets (PF, PFL and CaS) could have promising effects on methane reduction.

Ammonia concentration in the diet without RPF was high in comparison with other diets. This showed that palm oil without RPF might have hindered the effective consumption of NH_3-N for microbial protein synthesis. However, in diets, PF, PFL and CaS the microbial activity in the incubation medium remained unchanged. According to Hidayah *et al.* (2014), the inclusion of RPF in meals did not raise the ammonia levels.

Total VFA concentration was numerically high in the diet with no RPF added compared with diets with RPF. This proved that RPF was inert in the incubation medium and, therefore, did not influence microbial activity or fibre digestion. Similarly, there was no influence of vegetable oils on IVDMD, pH, ammonia and levels of TFVA (Roy *et al*., 2017). On the contrary, Naik *et al.* (2009) observed a linear increase (P=0.025) in TVFA with RPF supplementation. TVFA concentration was highest in the diet without RPF followed by CaS. One of the possible reasons for both treatments could be the high C18:3 linolenic acid contents in these diets and there is a reported effect of C18:3 on high TVFA production (Hidayah *et al.*, 2014). Moreover, high TVFA production from the CaS diet could be because of calcium availability that might progress the growth of the rumen bacterial population and their activities which leads to an increase in feed fermentation ultimately resulting in high TVFA production.

In terms of propionate concentration, acetate -to-propionate ratio, and CH_4 generation, the PFL diet produced the highest values. That could be because the administration of PFL stimulated the growth of bacteria that produce propionate, which in turn increased the level of propionate. Higher propionate concentrations have been associated with increased H_2 use and decreased CH_4 emissions. This might be due to the propionate formation pathway being a ruminal metabolic pathway that uses H_2 (Moss *et al*., 2000). Consequently, there was a reduction in the bond of H_2 and CO_2 that caused an ultimate decrease in methane. In general, the inclusion of RPF in the fermentation medium did not influence rumen fermentation parameters including pH, CH_4, NH_3N and VFA concentration statistically. The findings are coherent with earlier studies (Adeyemi *et al.*, 2015; Roy *et al*., 2017).

The end product of biohydrogenation is stearic acid (C18:0), and the lower concentration of C18:0 in a diet without RPF suggested a partial biohydrogenation of C18:1n9, C18:2n6 and C18:3n-3 resulting the highest level of biohydrogenation intermediate C18:1 *trans* 11 compared to other treatment groups, hence these findings are supported by the study conducted by Jalč *et al.* (2009). The higher concentrations of CLA *trans*-10 *cis*-12 in three diets containing RPF compared to the diet without RPF could be because RPF might have lost some of the effectiveness with a proportion becoming disassociated ultimately increasing the concentration of CLA *trans*-10 *cis*-12. It was reported that incomplete biohydrogenation of C18:2n6 and C18:3n-3 (Lee and Jenkins, 2011), produced C18:1 *trans*-11, CLA *cis*-9 *trans*-11 CLA *trans*-10 *cis*-12 and other conjugated isomers while C18:1n9 (AbuGhazaleh *et al.*, 2005) produced C18:1 *trans*-11. Similar to the present study Vargas *et al.* (2017) found that oil supplementation in the diet increased CLA *cis*-9 *trans*-11 and decreased CLA *trans*-10 *cis*-12 concentrations. The reason for that could be the diet without RPF contained relatively higher concentrations of C18:2n-6 compared to other treatment groups and it is documented that C18:2n-6 is converted to CLA *cis*-9 *trans*-11 which is then hydrogenated to C18:1 *trans* 11 (Jenkins *et al.*, 2008). This explains the higher concentration of C18:1 *trans* 11 and CLA *cis*-9 *trans*-11 in the diet without RPF.

The diet without RPF showed a low level of biohydrogenation for C18:1n-9 and C18:3n-3 while the highest biohydrogenation was observed in the PF. Biohydrogenation level for C18:2n-6 was highest in PFL and lowest in CaS. Carriquiry *et al.* (2008) determined *in vitro* rates of biohydrogenation of four commercially available RPF and found one RPF effective to be utilized to enhance post-ruminal levels of PUFA.

CONCLUSION

PFL and diet without RPF increased *in vitro* cumulative gas production, and gas production kinetics, improved IVDMD and IVOMD but did

not affect total VFA and their molar proportions. However, in addition to that PFL produced the highest propionate concentration, lower acetate-to-propionate ratio, reduced methane production and increased biohydrogenation of C18:2n-6 and C18 UFA compared to the diet without RPF. Therefore, it is concluded that PFL could improve rumen fermentation without having detrimental effects on rumen fermentation. However, further research should be conducted on rumen microbiology to confirm the findings.

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