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In vitro the Oretic Evaluation on Ruminal Fermentation Performance by Varying Proportion Supplementation of Carthamus Tinctorius Meal/*Brassica napus* Seed with Sorghum Seed in Ovine Rations

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Abstract: Oleaginous plant by products are widely used to improve ruminant growth performance for high energy or protein composition, however, few article reports the suitable fit amount for oleaginous by products to the ruminant diets in a large supplementation range. Therefore, this research was conducted to evaluate the effect of Safflower meal (*Carthamus tinctorius* L., SFM)/Canola seed (*Brassica napus*, CAS) with sorghum seed in theoretic proportion (0, 25, 50, 75 and 100%, respectively) in a specific concentrate-roughage ratio of (1:1) on *in vitro* ovine rumen fermentation performance such as the kinetics of gas production, ammonia nitrogen (NH_3-N), pH, methane production (CH_4) and *In Vitro* Dry Matter Disappearance (IVDMD) of maize stover. The results showed that gas production performance and methane production were significantly decreased but IVDMD and pH were obviously increased with the increasing proportions of both SFM and CAS in concentrated feed. To take a consideration offermentation performance and environment/cost factors, our results suggest that most suitable supplementation dosageof canola seed and safflower meal in concentrated feed are from 25-50 and 25-75%, respectively. However, in practical production of completed feed, the supplementation amount of safflower meal/canola seed also depend on concentrate-roughage ratios.

Key words: Safflower meal, canola seed, gas production kinetics, methane, *in vitro* dry matter disappearance

INTRODUCTION

Safflower (*Carthamus tinctorius* L.) an annual herb of Compositae is often used to extract oil and reclaim new soils in low input and water requirements. Mexico is one of the main production region of safflower (FAO, 2011), its yield was about 2.5 mt ha^{-1} (Gilbert, 2008). Safflower seeds contain 33-60% hull and 40-67% kernel with less palatability (Baumler *et al*., 2006) and are widely employed to the diets of pet bird, dairy cows and rams (Smith, 1996; Sudhamayee *et al*., 2004). High supplementation level of safflower decreases animal performance and undecorticated/decorticated safflower meal have only 45 and 68% *in vivo* Organic Matter (OM) digestibility in ruminants (Goss and Otagaki, 1954; Dixon *et al*., 2003). Protein degradability of safflower meal with highly degradable protein (Walli, 2005) varies between 60% (Chandrasekharaiah *et al*., 2002) and 70% (Dixon *et al*., 2003) to mix with rapeseed, soybean or linseed meal in different ratios. In fact, high content of hull in safflower mealhampers the utilization efficiency of nutrients in ruminant (Chandrasekharaiah *et al*., 2002). However, safflower meal with low price and high protein has special superiority in the practical ruminant production.

Brassica napus (Canola) has been widely utilized as an energy and protein source in ruminant with high biological value (CP: 20-43%; oil: >40%) (Ebrahimi *et al*., 2009) as a protein resource widely used to replace other

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protein resource, e.g., soybean, cottonseed or corn gluten meals (Maxin *et al*., 2013). However, canola proteins are extensively and easily degraded by rumen microbes (Khorasani *et al*., 1993) and also contains many anti-nutritional substances which hamper the widespread utilization in ruminant. For instance, glucosinolates reduce feed intake, induce iodine deficiencies and depress fertility (Tripathi and Mishra, 2007), phytic acid and the hexaphosphate of myoinositol chelating minerals and amino acids to form insoluble precipitates to reduce digestibility of proteins (Al-Kaisey *et al*., 2003).

In vitro fermentation technique has widely been considered to be suitable for evaluating the contribution of rumen microbial fermentation in the overall digestion of ruminant (Gosselink *et al*., 2004; Getachew *et al*., 2005). The kinetics of gas production and dry matter disappearance (Menke and Steingass, 1988) has been successfully applied to ruminant digestive researches to evaluate nutritional values of feed and corresponding rumen microbial fermentation performance, providing some virtualinformation over *in vivo* or *in situ* Methods (Gosselink *et al*., 2004; Wulf and Sudekum, 2005).

The changeable extents of *in vitro* gas production kinetics, CH_4 production and fibre digestibility are related to the nature or form of oleaginous seed (extruded, pressed meal or whole unprocessed) and their interactions with the composition of the basal diet (Lerch *et al*., 2012). However, little data are available to elaborate the appropriate amounts of unprocessed canola seed and extracted safflower meal in ovine rations. Therefore, this research was designed to study the integral effect of a variety of proportion of SFM/CAS with sorghum seed on *in vitro* fermentation performance by gas production kinetics, NH_3-N concentration, pH, CH_4 production and *In Vitro* Dry Matter Digestibility (IVDMD) of maize stover.

MATERIALS AND METHODS

Experiment design: This experiment was conducted to study the effect of 0.5 g mixtures (canola seed, CAS or safflower meal, SFM with sorghum seed in a series of ratios (λ, %), e.g., 0, 25, 50, 75 and 100, respectively) and 0.5 g maize stover (which were put into a little bag) on kinetics of gas production, CH_4 production, NH_3-N concentration, pH and IVDMD of maize stover. The amount of CAS or SFM in total substrate as follow, we set the ratios (λ, %) of SFM/CAS in the concentrated feed composited with SFM/CAS with sorghum seed in a concentrate-roughage ratio (1:1):

$$f_{(SFM/CAS)}(\%, \text{substrate}) = \frac{\lambda_{(SFM/CAS)} \times 0.5\ g^{(mixture)}}{0.5\ g^{(MStover)} + \left[\lambda_{(SFM/CAS)} + (1-\lambda)_{(sorghum)}\right] \times 0.5\ g^{(mixture)}} \times 100 \quad (1)$$

$$\langle \lambda = 0\%,\ 25\%,\ 50\%,\ 75\%,\ 100\% \rangle$$

where, $f_{(SFM/CAS)}$ (%, substrate) represents the proportion of SFM/CAS in the whole fermentation substrate (represented the supplementation dosage in concentrated feed of ovine diets). The 0.5 g mixture was composition of SFM/CAS with sorghum seed in various ratios. MStover represents maize stover.

Animal donor and substrate material: Four adult male Pelibuey sheep with permanent rumen-fistula (body weight, 45±5.0 kg) were used as inoculum donor according to Mexican official standard (NOM-220-SSA1, 2002). Each sheep was housed individually and free access to the water and fed 1.0 kg feed and 0.5 kg smashed sorghum seed. The ingredients of feed was provided with 64.2% sorghum, 19.3% alfalfa hay, 4.6% canola meal, 2.9% canola oil, 5.0% sorghum seed, 2.0% molasses, 1.0% purified tallow and 1.0% urea with a declared composition of 87% dry matter, 13.9% crude protein, 12.1 MJ kg^{-1} total energy, 5.3% crude fat, 0.54% calcium, 9.5% acid detergent fiber and 21.4% neutral detergent fiber. The substrates (safflower meal, canola seed and sorghum seed and maize stover, 500 g in each) were obtained from the plant of Natural Research Institute of Forestry, Agricultural and Livestock in Queretaro, Mexico. Substrate constituent in each treatment was shown in Table 1. The dry matter, CP, ashes and energy of substrates in SFM and CAS treatments increased and ether extract in CAS increased but which in SFM had minor changed with high proportion of SFM/CAS, respectively. The data of digestible starch was from 64.34-69.7% (Souilah *et al*., 2014) in this study we selected 64% as the digestible starch content in sorghum and to calculate the digestible starch content according the different sorghum proportion in different substrate treatments.

***In vitro* rumen fermentation:** *In vitro* fermentation was carried out according to the description by Tang *et al*. (2008). At 0700 a.m. before feeding, total 600 mL of rumen liquid from four adult Pelibuey male sheep were obtained, mixed and strained through four layers of cheese-cloth into an Erlenmeyer flask filled with CO_2. The *in vitro* fermentation solution was prepared by mixing the particle-free sheep ruminal fluid with artificial saliva buffer solution (Menke and Steingass, 1988) in a proportion of 1:4 (v/v) at 39°C under continuous flushing with CO_2 for 30 min. This experiment were divided into two parts for *in vitro* fermentation, one part was conducted to determine the gas production kinetics while other

Table 1: Chemical constituents of substrates for *in vitro* fermentation

Substrates	Ratio	Dry matter (%)	Crude protein (%)	Ether extract (%)	Ashes (%)	Total energy (MJ kg^{-1})	Digestible starch (%)†
Sorghum	/	88.97±0.06	8.96±0.08	1.42±0.01	1.19±0.06	16.20±0.02	64.0
Safflower meal	/	94.18±0.03	21.72±0.13	0.33±0.02	3.85±0.13	17.84±0.04	-
Canola seed	/	91.43±0.03	14.84±0.12	31.86±0.57	3.96±0.02	26.49±0.07	-
Maize stover	/	95.88±0.12	3.42±0.02	/	6.48±0.01	16.30±0.01	-
Nutritional ingredients of concentrated feed composited of safflower meal with sorghum seed							
SFM1	100:0	89.85±0.81	8.99±0.05	1.42±0.00	1.17±0.04	16.19±0.01	64.0
SFM2	75:25	90.29±0.03	12.17±0.06	1.15±0.01	1.87±0.06	16.61±0.02	48.0
SFM3	50:50	91.59±0.01	15.35±0.08	0.87±0.01	2.53±0.08	17.02±0.03	32.0
SFM4	25:75	92.87±0.03	18.52±0.11	0.61±0.01	3.19±0.10	17.43±0.04	16.0
SFM5	0:100	94.18±0.03	21.72±0.13	0.34±0.01	3.85±0.12	17.84±0.04	0.0
Nutritional ingredients of concentrated feed composited of canola seed with sorghum seed							
CAS1	100:0	88.95±0.04	8.93±0.05	1.42±0.00	1.21±0.04	16.20±0.01	64.0
CAS2	75:25	89.60±0.04	10.45±0.07	8.98±0.09	1.90±0.02	18.79±0.01	48.0
CAS3	50:50	90.21±0.03	11.91±0.08	16.74±0.19	2.59±0.01	20.50±0.88	32.0
CAS4	25:75	90.82±0.03	13.37±0.10	24.40±0.29	3.27±0.02	23.07±0.90	16.0
CAS5	0:100	91.43±0.03	14.84±0.12	31.67±0.38	3.96±0.02	25.64±0.92	0.0

†The data of digestible starch was from 64.34-69.7% (Souilah *et al*., 2014) in this study we selected 64% as the digestible starch content in sorghum and to calculate the digestible starch content according the different sorghum proportion in different substrate treatments; SFM-n/CAS-n (among, n = 1, 2, 3, 4, 5) represent the proportion of Safflower Meal (SFM)/Canola Seed (CAS) in concentrated feed (composited of safflower meal/canola seed with sorghum seed in this study) as 0, 25, 50, 75 and 100%, respectively

part was incubated in a separated bottle to obtain samples for CH_4, NH_3-N and IVDMD, however, all the determined indexes obtained/determined at the similar condition.

Part 1: Substrates (1.0 g in total each) were incubated with 100 mL rumen buffer solution at 39°C in 4 replicates of each treatment for 72 h to determine the gas production kinetics with ANKOM gas determination equipment (ANKOM Technology Corp., Fairport, NY, USA).

Part 2: Other substrates (0.5 g in total each) were carried out to determine the pH, NH_3-N concentration and IVDMD of maize stover for 24 and 48 h. Fermentation solution (50 mL) were added into the pre-warmed bottles with 0.25 g mixture and 0.25 g maize stover which were previously weighted into a nylon bag (pore size, 52 μm; specific surface area, 44 cm^2/g) was little higher than previous report (Valentin *et al*., 1999) with 33 cm^2/g. Bottles were sealed with rubber stoppers and screw-on caps and incubated at 39°C in a constant temperature water bath oscillator for 24 and 48 h in 3 replicates of each treatment. The gas volume measured at 25°C and gas sample obtained in each at 24 and 48 h incubation. The fermentation terminated by swirling the bottles in ice, uncapped and then to determine pH value immediately, took out the nylon bag washed with deionized water for 4 times until the water was clear. Meanwhile, 1 mL fermentation liquid free of substrate and microbes mixed with 0.25 mL meta-phosphoric acid (25%; w/v) were stored in -20°C for the determination of NH_3-N concentration.

Chemical analysis: Feed samples were analyzed using the standard methods of AOAC (Cunniff, 1995) for DM (No. 967.03), crude protein (No. 984.13) and ether extract (No. 954.02), ash content (No. 942.05), total energy and are expressed inclusive of residual ash. NH_3-N concentration of incubation liquid collected at 24 and 48 h were measured by phenol-hypochlorite and ninhydrin colorimetric procedures described by Broderick and Kang (1980). The CH_4 concentration was determined by gas chromatography with column of HP-PLOT/Q (Length 30 m; I.D 0.530 mm; FILM: 40 μm; Cat, No. 19095P-QO4).

Calculations and data analysis: *In vitro* gas curves were fitted with Logistic-Exponential (LE_0) Model descripted by Wang *et al*. (2011, 2013) using NLREG Version 5.0 (Sherrod, 1995):

$$V = V_F \cdot \frac{1-\exp(-\kappa \cdot t)}{1+\exp(b-\kappa \cdot t)} \times 100 \quad (2)$$

Where:

V = The final asymptotic gas production (mL/g) at time point t

V_F = The final asymptotic gas volume with dimension of 'mL'

κ = The fractional rate of gas production with dimension of '/h'

b = Shape parameter without dimension

Initial Fractional Rate of Degradation (FRD_0), Rate of Gas production (RG_t), half time ($t_{0.5}$) and fractional rate of gas production at half-life ($\mu_{0.5}$) proposed by Wang *et al*. (2013) as follows:

$$FRD_0 = \frac{\kappa}{1+\exp(b)} \quad (3)$$

$$RG_t = V_F \cdot \frac{\kappa \cdot (1+\exp(b)) \cdot \exp(-\kappa \cdot t)}{(1+\exp(b-\kappa \cdot t))^2} \quad (4)$$

$$t_{0.5} = \frac{\ln(2+\exp(b))}{\kappa} \quad (5)$$

$$\mu_{0.5} = \frac{\kappa \cdot (d+0.5)}{1+d} \quad (6)$$

Statistical analyses and Pearson correlations were performed using the GLM and CORR procedures of SAS (2001) 9.0, respectively and means within standard errors were compared with least squares means. Least squares means were reported throughout the text and statistical significances were declared if $p<0.05$.

RESULTS

Kinetics of *in vitro* gas production: The shape and final asymptotic gas production of different proportion of SFM or CAS treatments were obvious different in macroscopic as shown in Fig. 1, the changeable of gas cumulative production was minor from 48-72 h. V_F ($p<0.05$, Quadratic), k, FRD_0, RG_t and $\mu_{0.5}$ ($p<0.001$, Linear) of SFM treatments were decreased with increasing proportions of safflower meal in whole *in vitro* fermentation system yet $t_{0.5}$ ($p<0.001$, Linear) was increased with that of SFM (Table 2).

For canola seed, V_F and RG_t ($p<0.001$, Quadratic), $t_{0.5}$ ($p<0.05$, Quadratic) were decreased with increasing proportions of CAS (Table 3). Meanwhile, k ($p<0.001$, Quadratic), FRD_0 ($p<0.01$, Cubic) with that value of CAS 5 significant lower than others, $\mu_{0.5}$ ($p<0.001$, Quadratic) were increased with increasing proportions of CAS. However, the shape of gas production curve (b) ($p<0.05$, Cubic) was increased with increasing proportions of CAS.

NH_3-N, pH, CH_4 and IVDMD of maize stover: In this study, the methane production of different proportions of CAS ($p<0.001$, Cubic) and SFM ($p<0.001$, Cubic) were decreased with the increasing proportions of CAS and SFM at 24 h of *in vitro* fermentation time and that of CAS ($p<0.05$, Quadratic) and SFM ($p<0.001$, Linear) were also decreased with increasing ratios of canola seed or safflower meal at 48 h of *in vitro* fermentation (Fig. 2). In addition, methane production at 48 h was higher than that at 24 h while there was statistically insignificant difference between SFM1 and SFM2 at 48 h in addition of CAS1 and CAS2 at 48 h.

IVDMD of maize stover pushed into a nylon bag of CAS treatment ($p<0.01$, Linear) and SFM treatment ($p<0.001$, Linear) were increased with the increasing

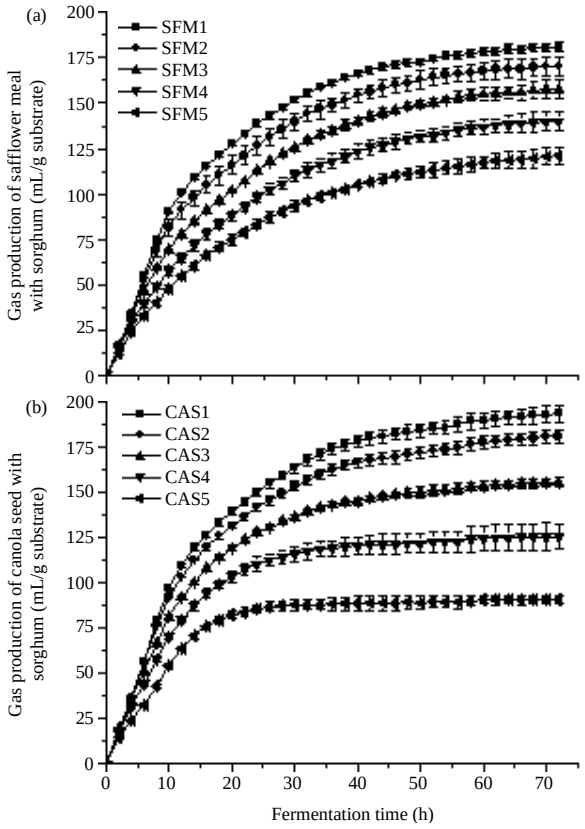

Fig. 1: a, b) Gas production dynamic curve of SFM-n or CAS-n in concentrated feed (composited of SFM/CAS with sorghum seed) with maize stover (ratio, 1:1) as substrate; SFM-n/CAS-n (among, n = 1, 2, 3, 4, 5) representthe proportion of Safflower Meal (SFM)/Canola Seed (CAS) in concentrated feed (composited of safflower meal/canola seed with sorghum seed in this study) as 0, 25, 50, 75 and 100%, respectively

proportion of canola seed and safflower meal at 24 h (Fig. 2, Table 4), however, IVDMD of maize stover in CAS ($p<0.05$, Quadratic) and SFM treatment ($p<0.05$, Cubic) were increased with both proportion of CAS and SFM with Sorghum at 48 h (Table 5). In addition, this study showed IVDMD in CAS 5 was higher than others.

NH_3-N concentrates were range from 33-66 mg dL^{-1}, NH_3-N concentration in CAS treatments at 24 h ($p<0.01$, Linear) (Table 4) and 48 h ($p<0.01$, Cubic) (Table 5) were changeable with increasing proportions of canola seed. For safflower meal treatment, NH_3-N concentration at 24 h ($p<0.001$, Cubic) and 48 h ($p<0.05$, Cubic) were varied with increasing proportions of safflower meal in substrate. Meanwhile that of high contents of SFM or CAS were higher than that of low contents in rumen *in vitro* fermentation liquid.

Table 2: Gas parameters of safflower meal with sorghum and maize stover on *in vitro* fermentation system (1 g substrate)

Treatments	$V_{F\ (mL/g)}$	k (%/h)	b	FRD_0 (%/h)	RG_i	$t_{0.5}$	$\mu_{0.5}$ (%/h)
SFM1 (0%)	182.2±2.32^{a}	6.1±0.06^{a}	-22.0±1.23	6.1±0.06^{a}	10.5±0.08^{a}	11.3±0.15^{b}	6.3±0.12^{a}
SFM2 (25%)	172.1±4.20^{b}	5.8±0.23^{b}	-24.3±1.70	5.8±0.23^{b}	9.4±0.49^{b}	12.0±0.44^{b}	5.9±0.17^{b}
SFM3 (50%	161.2±4.18^{c}	5.2±0.21^{c}	-23.3±3.68	5.2±0.21^{c}	7.9±0.12^{c}	13.4±0.55^{a}	5.3±0.26^{c}
SFM4 (75%)	144.2±4.05^{d}	4.9±0.12^{cd}	-26.3±4.34	4.9±0.12^{cd}	6.7±0.26^{d}	14.3±0.29^{a}	5.0±0.12^{c}
SFM5 (100%)	124.5±4.56^{e}	4.7±0.26^{cd}	-23.6±3.42	4.7±0.26^{cd}	5.5±0.20^{e}	14.9±0.79^{a}	4.8±0.26^{cd}
SEM	2.277	0.111	1.798	0.111	0.157	0.287	0.115
p-values							
Linear	***	***	NS	***	***	***	***
Quadratic	*	NS	NS	NS	NS	NS	NS
Cubic	NS	NS	NS	NS	NS	NS	NS

SFM-n (among, n = 1-5) represent the proportion of safflower meal in concentrated feed (composited of safflower meal with sorghum seed in this study) as 0, 25, 50, 75 and 100%, respectively; V_F: Final asymptotic gas volume with dimension of 'mL'; k: fractional rate of gas production with dimension of '%/h'; ^{b}Shape parameter without dimension; FRD_0: Initial Fractional Rate of Degradation; RG_i: Rate of Gas production, $t_{0.5}$: The half time at which half of the final gas production; $\mu_{0.5}$: Fractional rate of gas production at half-life. The significant difference was labeled within different proportions of CAS or SFM with sorghum and treatment effects were declared significance if p<0.05; *p<0.05; **p<0.01; ***p<0.001; NS: p>0.10; Ω: p<0.10; values are expressed as mean±SE

Table 3: Gas parameters of various proportions of canola seed in concentrated feed on *in vitro* fermentation system (1 g substrate)

Treatments	$V_{F\ (mL/g)}$	k (%/h)	b	FRD_0 (%/h)	RG_i	$t_{0.5}$	$\mu_{0.5}$ (%/h)
CAS1 (0%)	193.5±4.53^{a}	6.5±0.20^{c}	-15.8±10.92^{ab}	6.4±0.12^{c}	11.6±0.97^{a}	10.8±0.26^{a}	6.5±0.15^{c}
CAS2 (25%)	178.0±3.78^{b}	6.6±0.21^{c}	-20.5±2.12^{b}	6.6±0.21^{bc}	11.2±0.12^{a}	10.4±0.30^{a}	6.6±0.21^{c}
CAS3 (50%)	155.1±2.74^{c}	7.6±0.76^{c}	-8.5±9.96^{ab}	7.0±0.26^{ab}	10.1±0.36^{b}	9.7±0.10^{b}	7.3±0.26^{c}
CAS4 (75%)	124.3±6.72^{d}	10.7±1.61^{b}	-0.8±0.54^{a}	7.1±0.23^{a}	8.6±0.54^{c}	8.6±0.30^{c}	8.9±0.65^{b}
CAS5 (100%)	90.0±3.13^{e}	17.4±1.99^{a}	0.7±0.25^{a}	5.9±0.31^{d}	5.6±0.20^{d}	7.9±0.29^{d}	11.6±0.90^{a}
SEM	2.546	0.694	3.858	0.135	0.18	0.152	0.301
p-values							
Linear	***	***	**	NS	***	***	***
Quadratic	***	***	NS	***	***	Ω	***
Cubic	NS	NS	Ω	**	NS	NS	NS

CAS-n (among, n = 1, 2, 3, 4, 5) represent the proportion of Canola Seed (CAS) in concentrated feed (composited of seed with sorghum seed in this study) as 0, 25, 50, 75 and 100%, respectively; V_F: Final asymptotic gas volume with dimension of 'mL'; k: fractional rate of gas production with dimension of '%/h'; ^{b}Shape parameter without dimension; FRD_0: Initial Fractional Rate of Degradation; RG_i: Rate of Gas production; $t_{0.5}$: The half time at which half of the final gas production; $\mu_{0.5}$: Fractional rate of gas production at half-life. The significant difference was labeled within different proportions of CAS or SFM with sorghum and treatment effects were declared significance if p<0.05; *p<0.05; **p<0.01; ***p<0.001; NS: p>0.10; Ω: p<0.10; values are expressed as mean±SE

Table 4: The fermentation characteristics of different ratios of safflower meal, canola seed with sorghum at 24 h

	Different ratios of SFM and CAS† (%)								
Treatments	0	25	50	75	100	SEM	Linear	Quadratic	Cubic
CH_4 production (µmol/g substrate)									
Canola seed	45.80±0.57^{a}	34.30±1.640^{b}	33.50±1.29^{bc}	31.6±0.490^{c}	29.3±0.300^{d}	0.579	***	***	***
Safflower meal	45.80±0.57^{a}	43.60±1.460^{a}	38.10±1.19^{b}	36.6±0.430^{b}	19.8±0.430^{c}	0.532	***	***	***
DMD of maize stover (g kg^{-1})									
Canola seed	230.00±1.78	227.00±10.19	234.70±9.47	245.3±11.22	251.4±9.820	5.287	**	NS	NS
Safflower meal	230.00±1.78^{b}	248.20±8.280^{b}	269.60±7.39^{b}	269.7±16.06^{b}	297.8±25.89^{a}	8.385	***	NS	NS
NH_3-N of fermentation liquid (mg/100 mL)									
Canola seed	33.10±1.36^{d}	38.90±0.290^{c}	44.00±0.91^{b}	48.2±3.410^{b}	57.3±0.240^{a}	0.987	***	NS	NS
Safflower meal	33.10±1.36^{b}	32.70±0.620^{b}	41.50±1.53^{a}	43.3±0.280^{a}	40.6±0.870^{a}	0.598	***	***	***
pH value of fermentation liquid									
Canola seed	5.81±0.05^{e}	5.97±0.020^{d}	6.11±0.01^{c}	6.23±0.01^{b}	6.30±0.02^{a}	0.015	***	**	NS
Safflower meal	5.81±0.05^{e}	6.01±0.050^{d}	6.14±0.03^{c}	6.30±0.01^{b}	6.38±0.03^{a}	0.021	***	Ω	NS

Table 5: The fermentation characteristics of different ratios of safflower meal, canola seed with sorghum at 48 h

	Different ratios of SFM and CAS† (%)								
Treatments	0	25	50	75	100	SEM	Linear	Quadratic	Cubic
CH_4 production (µmol/g substrate)									
Canola seed	48.0±0.28^{a}	47.5±0.89^{a}	40.5±1.93^{b}	39.0±2.00^{bc}	31.2±1.040^{d}	0.800	***	Ω	NS
Safflower meal	48.0±0.28^{a}	46.1±2.57^{a}	43.4±0.98^{b}	40.0±2.46^{c}	35.7±1.700^{d}	1.055	***	NS	NS
DMD of maize stover (g kg^{-1})									
Canola seed	301.4±15.80^{b}	304.3±1.79^{b}	314.2±4.88^{b}	324.3±11.79^{b}	349.1±11.19^{a}	5.997	***	Ω	NS
Safflower meal	301.4±15.80^{d}	323.5±18.51^{c}	366.1±18.10^{b}	414.0±3.15^{a}	419.7±21.25^{a}	9.597	***	NS	Ω
NH_3-N of fermentation liquid (mg/100 mL)									
Canola seed	50.1±2.35^{b}	40.7±1.68^{c}	50.0±6.66^{b}	62.4±0.72^{a}	66.3±0.300^{a}	1.892	***	**	**
Safflower meal	50.1±2.35^{b}	51.9±5.24^{b}	42.3±4.82^{d}	47.5±5.77^{c}	57.8±4.190^{a}	2.672	NS	**	Ω
H value of fermentation liquid									
Canola seed	5.74±0.05^{e}	5.91±0.01^{d}	6.05±0.03^{c}	6.15±0.01^{b}	6.24±0.02^{a}	0.015	***	**	NS
Safflower meal	5.74±0.05^{e}	5.93±0.03^{d}	6.07±0.01^{c}	6.16±0.00^{b}	6.29±0.03^{a}	0.016	***	*	NS

†The values were expressed as an average with standard error within 3 replicates; the significant difference was labeled within different proportions of CAS or SFM with sorghum and the treatment effects were declared significance if p<0.05; *p<0.05; **p<0.01; ***p<0.001; NS: p>0.10; Ω: p<0.10

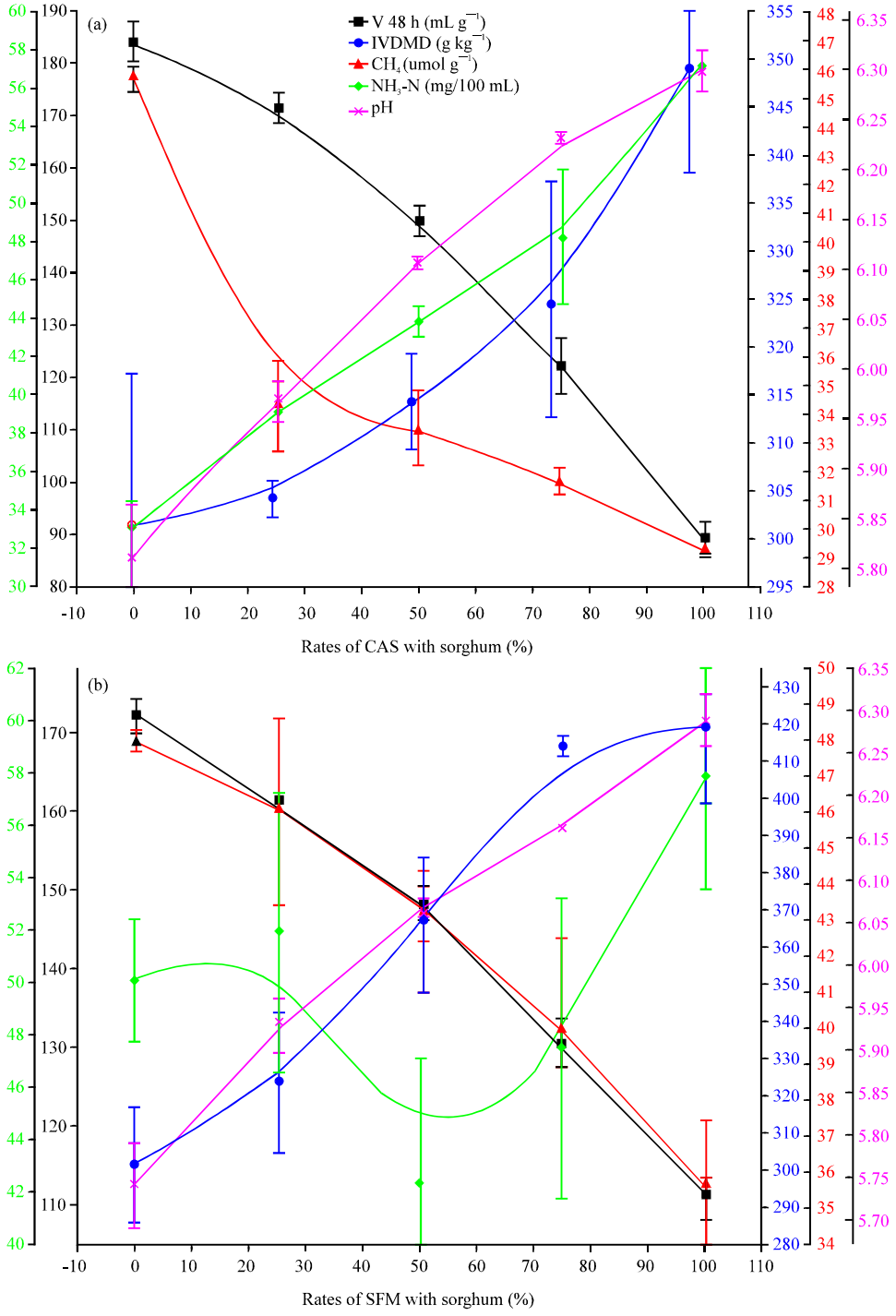

Fig. 2: a, b) Values tendency of fermentation parametersunder different ratios of SFM/CAS in concentrated feed fixed ratio with maize stover (1:1) *in vitro* fermentation at 48

The pH of all treatment were higher than 5.5 (Table 4 and 5), pH value of CAS treatment ($p<0.01$, Quadratic) and SFM treatment ($p<0.05$, Quadratic) at 24 h fermentation were increased with the increasing content of CAS and SFM. The changeable of pH value of CAS ($p<0.01$, Quadratic) and SFM ($p<0.01$, Quadratic) at 48 h were similar with that of 24 h however that of 48 h was lower than that of 24 h for each single treatment.

Table 6: The Pearson correlation coefficients of different chemical ingredients with fermentation performance for SFM and CAS at 24 and 48 h

Groups	CP	EE	Starch†	V_F‡	CH_4	NH_3-N	IVDMD	pH
CP		0.879	-0.835	-0.818	-0.638	0.256	0.537	0.856
EE	0.879		-0.473	-0.461	-0.329	-0.071	0.510	0.543
Starch	-0.835	-0.473		0.977	0.801	-0.550	-0.404	-0.958
V_F	-0.818	-0.461	0.977		0.802	-0.558	-0.396	-0.919
CH_4	-0.638	-0.329	0.801	0.802		-0.225	0.048	-0.831
NH_3-N	0.256	-0.071	-0.550	-0.558	-0.225		0.515	0.356
IVDMD	0.537	0.510	-0.404	-0.396	0.048	0.515		0.240
pH	0.856	0.543	-0.958	-0.919	-0.831	0.356	0.240	

CP: Crude Protein; EE: Ether Extract; V_F: Final asymptotic gas volume; NH_3-N: Ammonia nitrogen; IVDMD: *In Vitro* Dry Matter Disappearance; †The data of digestible starch was from 64.34-69.7% (Souilah *et al.*, 2014). ‡The number of V_F was 30; n: Analysis number within all of the treatments and repeats

Pearson correlation coefficients: V_F had significant correlation with CP (r = -0.818, negative) and starch (r = 0.977, positive); methane production had high correlation with CP (r = -0.638), starch (r = 0.801) and V_F (r = 0.802) (Table 6); IVDMD of maize stover had positive relation with CP (r = 0.539), energy (r = 0.535) and negative relation with starch contents (r = -0.404). Value of pH had high positive correlation with DM, CP (r = 0.856), energy and EE but had negative correlation with starch (r = -0.958) and V_F (r = -0.919).

DISCUSSION

The statistical differences in gas kinetic among SFM and CAS treatments could be due to the proportion and nature of their composition (Rubanza *et al*., 2003). High content of soluble carbohydrates could be due to one factor resulting to higher gas production in high sorghum proportion treatments (Zerbini *et al*., 2002; Amer *et al*., 2012). Meanwhile, some hull or anti-nutritional compositions in safflower meal or canola seed could also give rise to lower *in vitro* gas production parameters (V_F, RG_t and $t_{0.5}$ in this study) such as matairesinol-β-glucoside, 2-hydroxyarctiin-β-glucoside (Jin *et al*., 2010) and cyanogenic glucoside (Satish and Shrivastava, 2011) in safflower meal and sinapine, tannins, phytic acid (Brand *et al*., 2008) and glucosinolates (Bell, 1993) in canola seed.

V_F of SFM treatments was decreased with its ratio increasing in substrates that could be due to its the combination effects of non-digestible structural carbohydrate-hull improvement (Baumler *et al*., 2006), absolute high soluble carbohydrate reducing (Lechartier and Peyraud, 2011) as well as the anti-nutritional factors (such as cyanogenic glucoside (Satish and Shrivastava, 2011)). Some parameters of gas production (k, $FRDF_0$, RGt and $\mu_{0.5}$) were decreased while half time ($t_{0.5}$) was increased with high proportion content of safflower meal in the mixed substrate had further improved the most important factor influencing kinetics of gas production was the high soluble carbohydrate contents (Souilah *et al*., 2014). However, the changeable of FRD_0, k, $t_{0.5}$, Rg_t and $\mu_{0.5}$ implied SFM have some anti-nutritional or undigested ingredientsdecreased the efficiency of digestibility because of the hydration, removal of digestion inhibitors and/or attachment of microbes with substrate (Mertens, 1993) increased adsorption time, obviously those process should be more difficulty at occurring in structural carbohydrate (Tan *et al*., 2002). $t_{0.5}$ increased but $\mu_{0.5}$ decreased could be due to SFM's hull impede the attachment, adhesion, colonization and degradation processes of ruminal microorganisms (Varga and Kolver, 1997). Furthermore, the value of b (negative, -22.0 to -26.3 for all of SFM) reflected all of the synthetic gas production were the typical parabolic curve without lag time, resulted to no difference between fractional rate of gas production (k) and initial Fractional Rate of Degradation (FRD_0).

For CAS, V_F was the same reason as mentioned before. The gas parameters of k and FRD_0 in CAS were increased while V_F decreased, it seemed meaningless but high oil content in canola seed made it more reasonable as to some fatty acids have the potential to change the microbial ecosystem and the activity of some bacterial adhesion ability (Benchaar *et al*., 2008) or due to the easily degraded carbohydrate, some unknown nutritional factors from CAS to increase the initial fermentation performance. Those factors could activate some rumen bacterial bioactivity in the beginning period of fermentation but V_F have strong relationship with non-structural digestible carbohydrate (Tan *et al*., 2002). The curve shape b was increased with the increasing proportion of canola seed which means the asymptotic gas production was like as s curve with fermentation time accompany with a lag time. We speculate the ether extract, protein and energy of feed are incorrect way to judge the nutritional functions of feed for ruminant under the especial function of rumen microorganisms.

Rumen microorganism digests macromolecular of protein, carbohydrates and long chain polyunsaturated fatty acids to generate gas production, Volatile Fatty Acids (VFAs), secondary metabolites, microbial protein (Baba *et al*., 2002; Camacho *et al*., 2010). Beneficial diets added to the rumen results in an inhibition of deamination

and methanogenesis, resulting in lower NH_3-N, CH_4 and acetate and in higher propionate and butyrate concentrations (Calsamiglia *et al*., 2007). Microbial protein and feed protein are insufficient to supply adequate amounts of amino acids for optimal growth performance of ruminant (Kung Jr. and Rode, 1996), so NH_3-N as non-protein nitrogen or degradation metabolites of protein also plays an important role in keeping nutritive equilibrium of ruminant. In this study, NH_3-N were range from 33.1-66.3 mg dL^{-1} and were close to previously reports (Khorasani *et al*., 1989; Cherdthong and Wanapat, 2013), changeable of NH_3-N could be due to the different ratios of feed protein and the balance of microbial synthesis and degradation; NH_3-N at 48 h were a bit higher than 24 h implied the rate of microbial protein digested were faster than that of synthesis accumulation in 48 h incubation.

Rumen pH always reverses with concentrate ratios (Pina *et al*., 2009). The pH value which could be deemed as an important determinant of *in vitro* fermentation was range from 5.5-7.5 (Yuan *et al*., 2010), highly correlated with volatile fatty acid amounts, the population and activities of microbes, e.g., most acids like lactic acid which inhibits microbial activity at high concentration when pH is low, presumably due to greater penetration of cell membranes by lactic acid in non-ionized than in ionized form, protozoal population which are responsible for about 25% of rumen microbial cellulolytic and help maintain a higher pH by engulfing starch granules (Mould *et al*., 2005). In this study showed that pH was higher than 5.5 as to 50% fiber content (maize stover) added to fermentation system, similar with anterior research work (Poulsen *et al*., 2012). When ruminal pH is below to 5.5, rumen and gastrointestinal function are usually abnormal due to acidosis and many ruminal microbes cease growing despite an ability to survive even higher concentrations of H^+. In addition, the changeable of pH value was similar with reported by Russell (1998) when a cow, fed high concentrates, lower ruminal pH as to the starch-rich diets compared to forage based diets.

IVDMD of maize stover increased with increasing proportion of CAS and SFM could be due to the high starch content in sorghum reduced pH value of fermentation liquid and increase the production of short volatile fatty acid (Lechartier and Peyraud, 2011), further to effect the fibrolytic activity. This was consistency with high concentrate portion decreases the apparent crude fibre digestibility (Flachowsky and Schneider, 1992). It also implied that digestible rumen protein should be the major restricted factor for fibre digestibility when addition of sufficient soluble carbohydrate. During adaptation to a high-concentrate diet, pH exerts selective pressure against microbes intolerant of a low pH value. As pH drops, amylolytic and acid-tolerant bacteria increase while cellulolytic microbes decrease, excessive Non Structural Carbohydrate (NSC) may depress the energy available from propionic and lactic acid production reduce microbial protein synthesis (Tan *et al*., 2002) and decrease fiber digestibility as well as cause abnormalities in rumen tissue which may lead to ulcers and liver abscesses in animal (Ishler, 1996).

High starch diets as well as addition of lipid are the alternative methods to lower enteric CH_4 production (Beauchemin *et al*., 2009) which is consistency with high ether extract content of CAS significantly decrease the methane production in our study. In addition, as starch degradation in the rumen, pH drops to <5.5 due to overgrowth of starch-fermenting lactate-producing *Streptococcus bovis* and *Lactobacillus* sp. which is another factor to inhibit methanogenic activity for reducing methane production (Poulsen *et al*., 2012). However, pH value in our study was range of 5.6-6.4 corresponding with optimum for methanogenic activity in the pH range of 6.0-6.5 (Jarvis *et al*., 2000), so this could not be the major reason to reduce CH_4 for SFM and CAS, we speculated the high content of hull from SFM, oils from CAS or lower degradability of substrate could be due to reduce methane production in this study.

CONCLUSION

This research is a rare but valuable result to supplementary the system information (firstly, large range, from 0-100% usage amount in concentrated feed; secondly, different type: extracted meal and whole seed) of an oleaginous plant by-product used in practical ruminant production by mimicking the rumen digestive characteristics *in vitro*. Our results show that high proportion of safflower meal in concentrated feed have potential to lower *in vitro* gas production performance and methane production but increase the IVDMD of maize stover and pH while high proportion of canola seed obvious reduce *in vitro* gas production, methane production but increase IVDMD of maize stover. In addition, considering of fermentation performance and environment factor, the most suitable usage dosageof canola seed and safflower meal in concentrated feed are from 25-50 and 25-75%, respectively. However, in practical production of completed feed, the supplementation amount of safflower meal and canola seed are not only depend on those proportions in concentrated feed mentioned before but also on ratio of these concentrated feed with forage for ruminants. Therefore, our group suggest further research work might continue to discover

the suitable fit proportions of whole corresponding concentrated feed (containing a certain of canola seed/safflower meal) with other forage (corn stover, rice straw, alfalfa, etc.) by *in vitro* fermentation technique on rumen micro-niche balance performance.

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