Available online at www.ijmras.com

INTERNATIONAL JOURNAL OF MULTIDISCIPLINARYRESEARCH AND STUDIES

ISSN: 2640-7272

Volume:04; Issue:12 (2021)

Doi:10.33826/ijmras/v04i12.5

PAPAYA LEAF JUICE EXTRACT BY USING METHANOL

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ARTICLE INFO

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ABSTRACT

Papaya leaf metabolic root pull (extract) (PLE) at a cluster of 0 (CON), 5 (LLE), 10 (MLE) and 15 (HLE) mg/250 mg dry matter (DM)with 30 mL buffered quid fluid were incubated for 24h to spot its effect on in vitro ruminal methanogenesis and ruminal bio hydrogenation (BH). Total gas production wasn't affected (P>0.05) by the addition of PLE compared to the CON at 24 h of incubation. Methane (CH4) production (mL/250mg DM) decreased (P<0.05) with increasing levels of PLE. The acetate to propionate ratio was lower (P<0.05) in MLE (2.02) and HLE (1.93) compared to the CON (2.28). Supplementation of the diet with PLE significantly (P<0.05) decreased the speed of BH of C18:1n-9 (oleic acid; OA), C18:2n-6 (linoleic acid; LA), C18:3n-3(linolenic acid; LNA), and C18 polyunsaturated fatty acids (PUFA) compared to CON after 24 h incubation, which resulted in higher concentrations of BH intermediates like C18:1t11 (vaccenic acid; VA), c9t11 conjugated LA (CLA) (rumenic acid; RA) and t10c12 CLA. Real-time PCR analysis indicated that the entire bacteria, total protozoa, Buty-rivibrio fibri solvents, and methanogen population in HLE decreased (P<0.05) compared to CON, but the whole bacteria and B.fibri solvents population were higher (P<0.05) in CON compared to the PLE treatment groups.

KEYWORDS

Bio hydrogenation, In-vitro gas production, Methanogenesis, Papaya leaf extract, Rumen fermentation

INTRODUCTION

Creatures, particularly ruminants, produce methane from anaerobic turmoil in their gastrointestinal tracts as a pathway for the disposal of metabolic hydrogen produced during microbial metabolism (Jayanegaraetal. 2011). CH4 accounts for 2-12 loss of salutary gross energy in ruminants and is a potent hothouse gas with a global warming implicit 23 times advanced than that of carbon dioxide (CO2) in enmeshing the heat (Bhatta etal. 2013). Thus, reducing ruminal CH4 not only improves the effectiveness of nutrient application but also helps to cover the terrain from global warming. There's a need for relating feed complements with implicit to modify rumen turmoil for enhancing the effectiveness of application of feed energy while dwindling rumen methanogenesis (Bhatta etal. 2012). The conversion of salutary polyunsaturated adipose acids (PUFA) to impregnated adipose acids (SFA) by ruminants, which is called ruminal bio hydrogenation (BH), has important health counteraccusations for mortal health. Transformation of PUFA to SFA results in increased accumulation of several hydrogenation interceders in ruminant meat and milk, similar as rumenic acid (RA) and vaccenic acid (VA), which are well known for theiranti-carcinogenic,anti-atherogenic andanti-oxidative health-promoting parcels (Durmicetal. 2008). Among the possible strategies to manipulate ruminal turmoil, some factory bioactive composites feel to be more effective in reducing rumen methanogenesis and rumen BH. Plant excerpts containing bioactive composites similar as essential canvases, tannins, saponins and flavonoids have been shown to ameliorate rumen metabolism, similar as dwindling methanogenesis and protein declination in the rumen, adding microbial protein product and protein inflow to the duodenum affecting specific groups of rumen microbial populations (Patra & Saxena 2011; Wallace 2004). These bioactive composites, which have antimicrobial conditioning, are meant for protection of the host factory against irruption by foreign patches including pathogenic microbes (Patra & Saxena 2011). Carica papaya which can be plant in all tropical countries and numerous tropical regions of the world has numerous health counteraccusations similar as reducing heart attack threat (Runnie etal. 2004) and stand up against dengue (Ahmad etal. 2011). Phenolic acids, which have potent antioxidant parcels, are shown in waterless-methanol excerpt of papaya splint (PLE) as the main emulsion (Canini etal. 2007). PLE also displayed antitumor exertion and immune modulatory goods on mortal excrescence cells and supplemental blood mononuclear cells (Otsuki etal. 2010). Different detergents (ethanol, methanol, ethyl acetate, acetone, chloroform, petroleum ether, hexane and water) of PLE has shown to have exertion against some microbial communities similar as bacteria (Escherichia coli) and fungus (Baskaran etal. 2012). In another study, ethanol excerpt of PL which contains of alkaloids, flavonoids, steroids and tannins showed exertion against pathogenic bacteria (E. coli and Klebsiella pneumoniae) (Yusha'uetal. 2009). The objects of the present study were to identify the effect of PLE on in vitro gas product, turmoil characteristics, microbial population and processes involved in ruminal BH and ruminal methanogenesis to drop the product of methane and to drop BH of adipose acids in the rumen as well.

MATERIALS

Plant material and extraction procedure: Samples of PL were accumulated from Malaysian Agricultural Research Development Institute (MARDI) in September 2013. For the birth of PL (Fig. 1), 250 g of PL greasepaint were uprooted by 10 volumes (v/ w) of 80 methanol, and the birth was repeated thrice as described by (Lee etal. 2009). The excerpts were filtered through Whatman sludge paper (No. 2), concentrated with a vacuum evaporator (Heidolph, Schwabach, Germany), and fully dried with a snap drier (Labconco, Kansas, MO, USA). The excerpts were saved in tightly closed plastic holders and stored at 80 °C until farther analysis. The chemical composition and adipose acid content of substrates used in this study are shown in Table 1. The phenolic composites in PLE are shown in Table 2.

Animals and rumen liquor sampling: All beast operation and slice procedures were approved by the University Putra Malaysia Animal Care and Use Committee. Four rumen fistulated scapegoats (Kajang crossbred) with an average body weight of 39 ±0.70 kg were used as rumen liquor benefactors. The scapegoats were fed doubly daily with a diet containing a fixed quantum of alfalfa hay (AH) and concentrate (5050, w/ w). Rumen liquor was tried before the morning feeding at 0830 hours from four scapegoats and placed incontinently in warm (39 °C) isolated steins under anaerobic conditions. In the laboratory, samples were pooled in equal proportions and strained through four layers of cheesecloth under anaerobic conditions and incontinently used.

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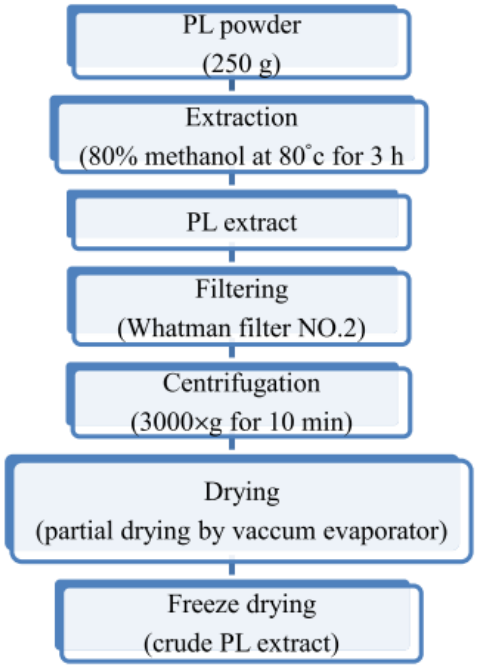

Figure 1 Schematic diagram depicting the extraction of papaya leaf in 80% methanol.

Table 1 Chemical composition and fatty acid content of substrates used for the *in vitro* incubations

(g/kg DM)	AH	Concentrate
DM	907.00	915.00
CP	203.00	167.00
NDF	517.01	244.03
ADF	334.00	117.00
EE	34.70	42.20
Fatty acid (g/100 g)		
C12:0	2.18	1.71
C14:0	1.61	1.63
C16:0	28.2	22.83
C16:1n-7	3.02	0.50
C18:0	7.44	4.72
C18:1n-9	5.40	16.40
C18:2n-6	42.59	43.92
C18:3n-3	9.56	8.29

ADF, acid detergent fiber; AH, alfalfa hay; CP, crude protein; C16:0, palmitic acid; C18:0, stearic acid; C18:1n-9, oleic acid; C18:2n-6, linolenic acid; C18:3n-3, alpha-linolenic acid; DM, dry matter; EE, ether extract; NDF, neutral detergent fiber; OA.

Table 2 Phenolic compounds in PLE

	TP (GAE/g)	TT (GAE/g)	CT (CTE/g)	HT (GAE/g)
PLE	30.31	28.36	17.39	10.96

CTE, catechin equivalent; CT, condensed tannin; HT, hydrolyzed tannin; GAE, gallic acid equivalent; PLE, papaya leaf extract; TP, total phenol; TT, total tannin.

METHODS

In vitro gas production: Gas Product was measured according to (Fievezetal. 2005), in which 30mL of softened rumen fluid result were allocated into 100 mL calibrated plastic hypes independently containing different attention of PLE, videlicet the CONTROL with no addition of PLE (CON, 0), 5 mg/ 250 mg dry matter (DM) (low splint excerpt LLE), 10 mg/ 250 mg DM (medium splint excerpt MLE) and 15 mg/ 250 mg DM (high splint excerpt HLE) which were original to 0, 20, 40 and 60 g/ kg DM, independently. Linoleic acid (LA) (Sigma-Aldrich Chemical Company).A softened rumen liquor only incubated for 24 h was used as a blank to calibrate the in vitro gas product system. Also, the treatment samples were taken before incubation (0 h) to determine the original adipose acid profile and after 24 h incubation to determine the BH of unsaturated adipose acids. Total gas product at 2, 4, 6, 8, 10, 12 and 24 h of incubation were estimated by relegation of the hype piston. Net gas product values were corrected by abating blank values for the sample's incubation.

Sampling and measurements after: After 24 h incubation, gas in the headspace was transferred into a gas slice bag (SKC Inc, Eighty Four, PA, USA) for analysis of methane by a gas chromatograph (Agilent 5890 Series Gas Chromatograph, Wilmington, DE, USA) equipped with a thermal conductive sensor (TCD). Estimation was completed using standard methane prepared by Scotty Specialty Feasts (Supelco, Bellefonte, PA, USA). The pH of the contents of the hypes was determined using a pH electrode (Mettler-Toledo Ltd., Leicester, UK). Also, 1mL of meta phosphoric acid was added to 4 mL of the incubated samples and centrifuged at 3000 × g for 10 min at 25 °C. Also,0.5 mL of clarified sample was added to0.5 mL of 4-methyl-n-valeric acid (20mmol/ L) before determining the attention of unpredictable adipose acids (VFAs), videlicet acetic, propionic and butyric acids using gas chromatography with a Quadrex 007 Series (Quadrex Corporation, New Haven, CT, USA) clicked phase fused silica capillary column (15m,0.32 mm internal periphery,0.25 μm film consistence) in an Agilent 7890A gas-liquid chromatograph (Agilent Technologies, Palo Alto, CA, USA) equipped with a honey ionization sensor (FID). Attention of NH3N (mg/ dL) was determined using the colorimetric system described by (Solorzano 1969). A standard wind was prepared to determine whether a direct relationship was between the varying attention of ammonium chloride (NH4Cl) standard result and the intensity of the color produced.

FATTY ACID ANALYSIS AND ESTIMATION OF BIOHYDROGENATION

Total adipose acids were uprooted from the whole hype content after 24 h of incubation grounded on the system of (Folch etal. 1957), modified by (Rajionetal. 1985) as described by (Ebrahimi etal. 2012) using chloroform/ methanol 21 (v/ v) containing butylated hydroxyl toluene to help oxidation during sample medication. After complete separation, the lower phase was collected in a round bottom beaker and rotary faded (Laborota 4000-effective; Heidolph) at 70 °C. An

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internal standard, heneicosanoic acid (C210) (Sigma Chemical,St. Louis, MO, USA), was added to each sample before trans methylation to determine the individual adipose acid attention within the sample. Trans methylation of the uprooted adipose acids to their adipose acid methyl esters (FAME) was carried out using potassium hydroxide in methanol and 14 methanolic boron trifluoride (BF3). The FAME were separated by gas chromatography (Agilent 7890A), using a Supelco SP 2560 capillary column of 100m ×0.25 mm internal diamter ×0.2 µm film consistence (Supelco, Bellefonte, PA, USA). One microliter was fitted by an bus sample (Agilent Auto Analyzer 7683 B series, Agilent Technologies, Santa Clara, CA, USA) into the chromatograph equipped with a split/ splitless injector and a FID. The carrier gas was nitrogen at a inflow rate of1.2 mL/ min. The split rate was 120 after injection of 1 µL of the FAME. The injector temperature was programmed at 250 °C, and the sensor temperature was 270 °C. The peaks of samples were linked, and attention calculated grounded on the retention time and peak area of known norms (Sigma Chemical). The adipose acid attention is expressed as the chance of total linked adipose acids.The BH of PUFA was premeditated as the drop of PUFA from the eccentric PUFA at time zero (0 h) of incubation as described by (Jayanegaraetal. 2012a) using the formula:

$$\mathrm{BH}\ (\%) = [PUFA(0h) - PUFA\ (24h) \div PU$$

Estimation of rumen microbial population using real-time PCR:The contents of the syringes were used to enumerate quid bacterial and protozoal population at the end of 24 h incubation. Three hundred micro liters of the quid liquor mixture were used for extraction of total DNA using the QIA amp® DNA Stool kit (Qiagen Gmbh, Hilden, Germany). The congregation of extracted DNA was measured by Nano drop 2000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA). The relative plenty of total bacteria, total methanogen for 1min, extension at 72°C for 90 s, and a final extension at 72°C for 10min. Real-time PCR was performed with the Bio-Rad CFX96 Touch (Bio-Rad Laboratories, Hercules, CA, USA) using 96- well optical grade plates. The PCR reaction was performed on a total volume of 25µL using the iQTMSYBR Green Super mix (Bio-Rad Laboratories, Hercules, CA, USA).To authenticate the specificity of amplification, a melting curve analysis was conveyed out after the last cycle of each amplification. The PCR primers used for amplifying target bacteria in the quid are shown in Table 3.

STATISTICAL ANALYSIS:

All experimental data were analyzed using SAS 2003. For statistical analysis of in vitro gas proffering, sampling at different times were added to the model and analyzed using repeated measures analysis of variance. The microbial data were conciliated using the log10-transformation for the investigation. Values of P< 0.05 were considered significant.

RESULTS AND DISCUSSION

EFFECT OF PLE ON GAS AND METHANE PRODUCTION

The effects of PLE on gas proffering characteristics and methane proffering are shown in Table 4. Progressive gas production of different PLE levels at different times of incubation is shown in Figure 2. Increases in levels of PLE did not affect total gas proffering (mL/250mg DM) (Fig. 2) in PLE treatment groups. In an in vitro study by (Kumar et al. 2011) (water, ethanol and methanol) fractions of Mangifera indica (mango), Eugenia jambolana (jamun), Aegle marmelos (bel), Zyzipus jujuba (ber), Azadirachta indica (neem) and Ficus religiosa (peepal) foliole, only the methanol extract of E. jambolana caused a reduction in gas proffering, and they attributed their results to the high condensed tannins compared to the other leaves. Results of the present study also suggest the presence of secondary metabolites (tannins) in the methanol extract of PL could not inhibit gas proffering among treatments. Methane proffering (mL/250 mg DM) decreased with the increasing levels of PLE (Table 4). Methane proffering at 24 h of incubation was 5.90, 5.13, 4.99 and 3.89 for CON, LLE, MLE and HLE, respectivelyin which their results are consistent with the methane reduction of HLE (34%) obtained in the current study. They also attributed their results to the tannin content of the foliole samples. The reduced quid CH4 production in the present study could also be linked to the presence of secondary metabolites such as tannin in PLE. In an in vitro study conducted by (Agarwalet al. 2011) with different solvent

Table 3 PCR primers used for amplifying target rumen bacteria and protozoa

Microorganism	Sequence 5′ – 3′	Reference
Total bacteria F^1	CGGCAACGAGCGCAACCC	(Koike & Kobayashi 2001)
Total bacteria R^2	CCATTGTAGCACGTGTGTAGCC	
Butyrivibrio fibrisalvens F^1	TAACATGAGTTTGATCCTGGCTC	(Liu *et al.* 2012)
Butyrivibrio fibrisalvens R^2	CGTTACTCACCCGTCCGC	
Total methanogen F^1	TTCGGTGGATCDCARAGRGC	
Total methanogen R^2	GBARGTCGWAWCCGTAGAATCC	(Zhang *et al.* 2008)
Total protozoa F^1	ACCGCATAAGCGCACGGA	
Total protozoa R^2	CGGGTCCATCTTGTACCGATAAAT	(Sylvester *et al.* 2004)

F^1, forward; R^2, reverse.

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Table 4 Effect of PLE on gas production and fermentation characteristics after 24 h *in vitro* incubation

Parameters	Experimental diets					*P*-value	
	CON†	LLE‡	MLE§	HLE¶	SEM	Linear	Quadratic
Total gas (mL/250 mg DM)ns	52.50	48.16	48.16	44.33	4.85	0.53	0.71
Rate (/h)ns	4.37	4.01	4.01	3.69	0.40	0.53	0.72
CH_4	5.90^{a}	5.13^{a}	4.99^{ab}	3.89^{b}	0.37	0.02	0.30
Ph	7.12	7.14	7.12	7.12	0.03	0.95	0.50
NH_3N (mg/dL)	17.69^{c}	18.27^{bc}	18.91^{a}	19.56^{a}	0.29	0.007	0.94
Acetic (mol/100 mol)	67.88	72.43	68.71	65.17	3.09	0.85	0.28
Propionic (mol/100 mol)	30.56	34.88	33.96	33.81	2.13	0.27	0.32
Butyric (mol/100 mol)	2.07	2.10	1.88	2.00	0.07	0.07	0.18
Total VFA (mmol/L)	100.52	109.43	104.56	100.99	5.18	0.58	0.29
Acetic /propionic ratio	2.28^{a}	2.07^{ab}	2.02^{b}	1.93^{b}	0.07	0.02	0.40

†CON: control (50% concentrate + 50% alfalfa hay), ‡LLE: 5 mg of papaya leaf methanolic extract (PLE) /0.25g dry matter (DM), §MLE: 10 mg of PLE / 0.25g DM, ¶HLE: 15 mg of PLE /0.25g DM, SEM: standard error of mean, ^{a,b,c}different letters in each row denote significant difference ($P < 0.05$), nsNot significant.

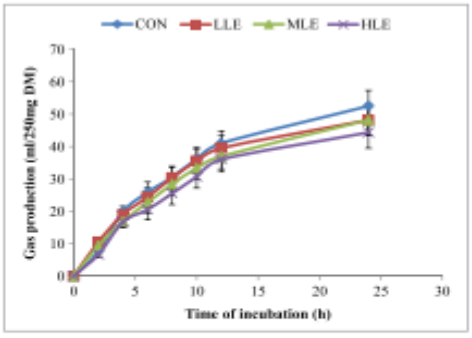

Figure 2 Cumulative gas production of different papaya leaf methanolic extract (PLE) levels at different times of incubation. CON: control without PLE (50% concentrate + 50% alfalfa hay); LLE: 5 mg of PLE /250 mg dry matter (DM); MLE: 10 mg of PLE / 250 mg DM; HLE: 68 mg of PLE /250 mg DM; SEM, standard error of mean. Vertical bars are standard error.

excerpts from berries of Sapindusmukorossi (cleaner nut), ethanol, methanol and water excerpts produced 96, .20 and 23 lower CH4, independently compared to the control group. They indicated that the ethanol excerpt of the berries of sapindusmukorossi was the most operative antimethanogenic agent compared to the other solvent excerpts. During the last decade, experimenters have shown. interest in using factory excerpts containing secondary metabolites (PSM) as feed complements to control CH4 product because of their antimicrobial and antiprotozoal conditioning (Halim etal. 2011; Kamra etal. 2012; Wischer etal. 2013) and also shift of short chain adipose acid composition down from acetate and hence less product of hydrogen (H2) and lower CH4 conformation. In the present study, the loftiest CH4 reduction was related to HLE with a-34 reduction compared to CON, which is harmonious with the results reported by (Kamra etal. 2006, .2008, 2009) about the goods of factory excerpts on quid. methanogenesis (in vitro). According to their results and from 93 factory excerpts, 20 factory excerpts displayed antimethanogenicexertion, with the loftiest inhibitory exertion related to ethanol excerpts followed by methanol and water excerpts, independently.

Effect of PLE on rumen fermentation parameters:The effect of PLE on turmoil characteristics after 24 h in vitro incubation is shown in Table 4. In the present study, the use of PLE didn't negatively affect quid turmoil parameters. Eliminations of PLE did not affect pH which equaled7.13 among the treatments. Total VFAs and molar proportions of acetic, propionic . and butyric acids were also not significantly (P>0.05) affected. The acetic propionic rate was advanced in CONVFAs are the end products of rumen microbial turmoil and represent the main force of metabolizable energy for ruminants. Thus, their reduced product would be nutritionally inimical for the ruminants (Busquetetal. 2006). Total VFA attention was not affected by7.5 mg/ kg DM of excerpts of garlic, cinnamon, yucca, anise, oregano or pepper compared with control (no excerpt) in a binary- inflow nonstop culture fermenters study by (Cardozo etal. 2004). They also suggested that those complements at mentioned bolusesthe result of microbial adaption to the excerpts. It is likely that the use of high boluses of factory excerpts with antimicrobial exertion would drop microbial exertion and diet fermentability. It appears that the different boluses of PLE (LLE, MLE and HLE) used in the present study weren't poisonous to the ruminal microbes. It should also be noted that the different results attained in other presence and position of factory secondary metabolites in the factory products Table 5.

Table 5 Concentration of fatty acids in rumen liquor at 0 h and 24 h of *in vitro* incubation

FA (g/100 g)		Experimental diets				SEM	*P*-value	
	Time	CON†	LPL‡	MPL§	HPL¶		Linear	Quadratic
C14:0	0 h	2.14	2.42	2.60	2.66	0.17	0.227	0.4244
	24 h	3.09	2.77	3.18	3.03	0.26	0.2749	0.4749
C16:0	0 h	15.44	18.23	18.53	18.77	1.06	0.1649	0.7324
	24 h	22.95	20.97	22.64	21.68	0.89	0.3176	0.8068
C16:1	0 h	1.05^{ab}	0.54^{b}	1.29^{a}	0.53^{b}	0.18	0.2018	0.0975
	24 h	0.58	0.82	0.76	0.65	0.13	0.2360	0.1234
C18:0	0 h	32.54^{b}	47.36^{a}	42.89^{a}	45.48^{a}	2.68	0.1995	0.0229
	24 h	57.02	56.41	53.54	52.72	2.56	0.3945	0.1259
C18:1 t11	0 h	0.35^{a}	0.14^{b}	0.14^{b}	0.14^{b}	0.06	0.0351	0.1843
	24 h	1.43^{b}	1.50^{b}	1.85^{ab}	1.96^{a}	0.13	0.0640	0.6777
C18:1n-9	0 h	18.23	16.49	18.32	17.71	1.49	0.5675	0.5659
	24 h	6.98	8.04	8.74	9.95	1.69	0.7155	0.7144
C18:2n-6	0 h	22.81^{a}	9.23^{b}	10.16^{b}	8.37^{b}	1.21	<0.001	0.0001
	24 h	3.00	3.23	3.27	3.35	0.67	0.0201	0.1140
C18:3n-3	0 h	2.70	2.67	2.72	2.72	0.14	0.1890	0.3323
	24 h	0.69^{b}	1.21^{a}	1.24^{a}	1.33^{a}	0.08	0.0432	0.7520
CLAc9t11	0 h	0.25	0.20	0.21	0.22	0.03	0.3020	0.6739
	24 h	1.39	1.46	1.58	1.70	0.10	0.7881	0.9132
CLAt10c12	0 h	0.15	0.13	0.12	0.14	0.02	0.6770	0.2507
	24 h	0.46^{b}	0.47^{b}	0.62^{a}	0.68^{a}	0.04	0.0040	0.6753
C20:4n-6	0 h	1.19^{a}	0.56^{b}	0.42^{b}	0.55^{b}	0.12	0.0003	0.5353
	24 h	0.57	0.70	0.55	0.59	0.05	0.0016	0.5990
C20:5n-3	0 h	1.20	0.65	1.06	1.08	0.22	0.9052	0.6142
	24 h	0.85	1.19	0.93	1.00	0.18	0.9067	0.6195
C22:5n-3	0 h	0.56	0.46	1.12	0.75	0.32	0.3022	0.4737
	24 h	0.65	0.70	0.59	0.80	0.09	0.2961	0.4682
C22:6n-3	0 h	1.35	0.87	0.38	0.60	0.41	0.2007	0.8365
	24 h	0.28	0.47	0.46	0.50	0.10	0.2160	0.8420
SFA	0 h	50.13^{b}	68.02^{a}	62.02^{a}	66.91^{a}	2.44	0.0416	0.0234
	24 h	83.07	80.16	79.37	77.45	2.38	0.2967	0.2435
MUFA	0 h	19.28	17.03	19.61	18.24	1.46	0.4877	0.4627
	24 h	7.56	8.86	9.49	10.61	1.75	0.6597	0.6411
n-6PUFA	0 h	24.00^{a}	9.79^{b}	10.58^{b}	8.93^{b}	1.21	<0.001	0.0001
	24 h	3.57	3.93	3.82	3.94	0.67	0.0153	0.1150
n-3PUFA	0 h	5.82	4.66	5.29	5.15	0.76	0.8421	0.8731
	24 h	2.48^{b}	3.59^{ab}	3.24^{ab}	3.64^{a}	0.36	0.0871	0.8964
C18 UFA	0 h	44.50^{a}	28.88^{b}	31.69^{b}	29.32^{b}	2.08	0.0338	0.2150
	24 h	13.98	15.94	17.32	18.99	2.22	0.2989	0.2582

†CON: control (50% concentrate + 50% alfalfa hay (AH)), ‡LLE: 5 mg of papaya leaf methanolic extract (PLE) /0.25g dry matter (DM), §MLE: 10 mg of PLE /0.25g DM, ¶HLE: 15 mg of PLE /0.25g DM; Saturated fatty acids (SFA) = C14:0+C16:0+C18:0. Monounsaturated fatty acids (MUFA) = sum of C14:1 +C16:1+C17:1+C18:1n-9. Polyunsaturated fatty acids (PUFA) n-3 = sum of C18:3n-3+C20:5n-3+C22:5n-3+C22.6n-3. PUFA n-6 = sum of C18:2n-6 +C20:4n-6. C18 PUFA: sum of (C18:3n-3+C18:2n-6+C18:1n-9). CON: control without PLE (50% concentrate + 50% AH), LLE: 5 mg of PLE /250 mg DM, MLE: 10 mg of PLE /250 mg DM, HLE: 15 mg of PLE /250 mg DM; ^{a,b}different letters in each row denote significant difference ($P < 0.05$).

Effect of PLE on rumen biohydrogenation:The outcome of BH of oleic acid (OA), linoleic acid (LA), . linolenic acid (LNA) and C18 PUFA are exhibited in Figure 3. The effect of PLE on the attention of adipose acids in quid liquor at 0 h and 24 h of in vitro incubation are shown in Table 6. The process of BH reduces the rumen exodus of PUFA and contributes to the accumulation of cis and trans isomers in ruminant products, involving conjugated linoleic acid (CLA) and trans monoenes (Jayanegaraetal. 2012b). Adding PUFA and CLA contents in ruminant products through nutritionally controlling BH has

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attracted important attention in recent times. Among recommended nutritive strategies, supplementing with phenolic composites seems .to play a major part in this respect (Jayanegaraetal. 2012a; Vasta etal. 2009). The results of the present study give conclusive substantiation for the influence of phenol containing PLE, anyhow of its specific nature. The in vitro BH of OA, LA, LNA and C18 PUFA were outstandingly different.

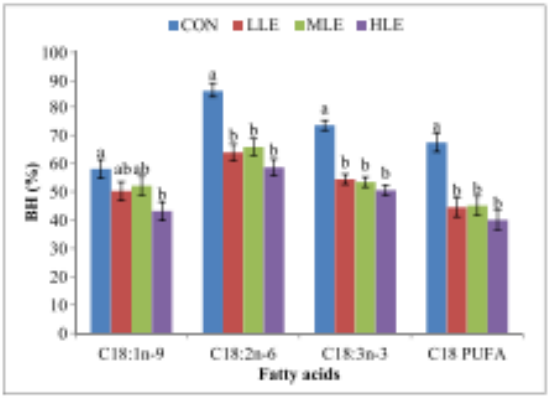

Figure 3 Effect of papaya leaf methanolic extract (PLE) on in vitro biohydrogenation (BH) rate (%).CON: control without PLE (50% concentrate + 50% alfalfa hay); LLE: 5 mg of PLE /250 mg dry matter (DM); MLE: 10 mg of PLE /250 mg DM; HLE: 68 mg of PLE /250 mg DM. Vertical bars are standard error, different letters denote significant difference ($P < 0.05$).

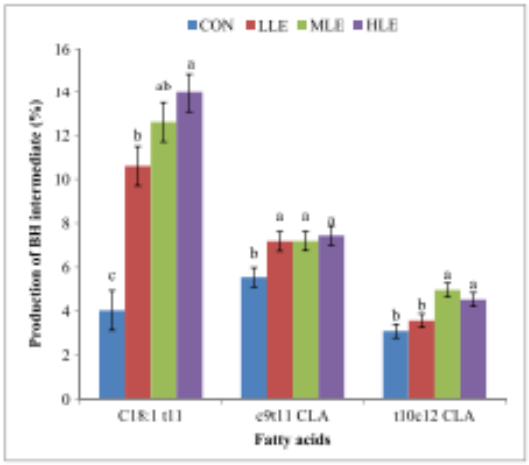

Figure 4 Effect of different levels of papaya leaf methanolic extract (PLE) on production of BH intermediate. CON: control without PLE (50% concentrate + 50% alfalfa hay); LLE: 5 mg of PLE /250 mg dry matter (DM); MLE: 10 mg of PLE /250 mg DM; HLE: 68 mg of PLE /250 mg DM. Vertical bars are standard error, different letters denote significant difference ($P < 0.05$).

Table 6 Effect of PLE on the population of bacteria and protozoa at 24 h *in vitro* incubation

Microorganism	Experimental diets				SEM	*P*-value	
	CON†	LLE‡	MLE§	HLE¶		Linear	Quadratic
Total bacteria	11.18^{a}	11.06^{a}	10.86^{b}	10.67^{b}	0.12	0.008	0.390
Methanogens	8.03^{a}	7.61^{ab}	7.54^{ab}	7.23^{b}	0.15	0.040	0.370
B. fibrisolvens	4.13^{a}	3.54^{b}	3.52^{b}	3.51^{b}	0.13	0.005	0.100
Total protozoa	5.64^{a}	5.48^{ab}	5.37^{ab}	5.20^{b}	0.10	0.070	0.850

†CON: control (50% concentrate + 50% alfalfa hay), ‡LLE: 5 mg of papaya leaf methanolic extract (PLE) /0.25g dry matter (DM), §MLE: 10 mg of PLE / 0.25g DM, ¶HLE: 15 mg of PLE /0.25gDM; SEM: standard error of mean; ^{a,b}different letters in each row denote significant difference ($P < 0.05$). The results are presented as $\log_{10}$ cells/L.

EFFECT OF PLE ON THE POPULATION OF BACTERIA AND PROTOZOA:

The relative abundance of total rumen bacteria, total methanogens, B. fibri solvens and total protozoa is presented in Table 6. In terms of total bacteria population had the best (P< 0.05) total bacteria numbers. The use of PLE within the diets affected the relative abundance of methanogenic bacteria and protozoa significantly (P< 0.05) compared with CON. The decrease within the total generally depresses protozoal populations. Ethanol, methanol and water extracts from the berries of Sapindusmukorossi (soap nut) exhibited 70-90% antiprotozoal activity and had lower protozoa count within the treated samples compared to the control in an in vitro condition (Agarwal et al. 2011). Plant extracts having PSM as their content might inhibit methane emission by directly inhibiting methanogens as these compounds have anti-microbial activities against different microbial groups and/or indirectly by inhibiting protozoal activity, resulting in a reduced number of methanogens (Kamra et al. 2012). Protozoa represents almost 1/2 the microbial biomass within the rumen. it's expected that reducing protozoa would also reduce methanogen populations because 10 to twenty of methanogens board association with protozoa, thus decreasing methane production (Wang et al. 2011). Interestingly, populations of total methanogens were affected (P< 0.05) by PLE, especially at higher concentrations (HLE).The population of B. fibrisolvens was significantly (P< 0.05) higher for CON (4.13) compared to fibri solvents is that the most active species and plays a serious role in hydrogenating unsaturated fatty acids to VA. The results obtained within the present study did not show a rise within the population of B. fibri solvents between PLE treatments and CON. These results suggest that PLE altered BH without changing the ruminal populations of B. fibri solvents.

CONCLUSION

Based on the info obtained from this study, it could be concluded that PLE didn't affect ruminal fermentation characteristics negatively, reduced BH of C18 PUFA and contributed to the assembly of VA and RA. the current study showed that PLE at different levels of inclusion reduced CH4 production without a negative effect on total gas production. However, more comprehensive in vivo studies with animal hosts need to be dispensed to guage the sustainability of PLE supplementation to mitigate CH4 production and rumen BH without deleterious effects on the animal as a whole.

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