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Evaluation of Potential New Opportunities for Herbal Plants as Natural Products on Rumen Fermentation Patterns *in vitro*

Research Article

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ABSTRACT

Two experiments (Exp.) were conducted to determine the effects of dietary addition of five herbal plants (HP) alone or as herbal plant mixture (HPM) on *in vitro* gas production and ruminal fermentation patterns. In Exp. 1, five varieties of HP (garlic, eucalyptus, cinnamon, thyme, and peppermint) were added to the diet at 3 different concentrations (5, 10 and 15% of concentrate dry matter). In Exp. 2, the HPM was used at the concentration of 2, 4 and 6%. The results show that most ruminal fermentation parameters were affected by HP and HPM. The addition of HP and HPM to the diet significantly increased total *in vitro* gas production (b) and the fractional rate constant of gas production (c, $P<0.01$). Concentrations of NH_3 (Exp. 1), CH_4 (Exp. 1 and 2), total volatile fatty acids (Exp. 1 and 2), acetate (Exp. 1), propionate (Exp. 1 and 2) and acetate/propionate ratio (Exp. 1) were affected by HP and HPM. Ruminal short-chain fatty acids (SCFA, $P<0.01$ and $P<0.05$ for 16 or 24 h, respectively) and organic matter degradability (OMD) were higher at 16 or 24 h of incubation with HP than control. However, dry matter degradability (DMD) and metabolizable energy (ME) of 6% HPM were lowered compared to control after 24 h ($P<0.003$ and $P<0.04$, respectively). The results point to the promising beneficial effects of HP and HPM in ruminant nutrition as rumen modifier to improve digestibility and nutrient utilization that may improve performance on *in vivo* conditions.

KEY WORDS digestibility, herbal plant, *in vitro* gas production, rumen fermentation, rumen modifier.

INTRODUCTION

Nutritional strategies to improve the production of ruminants have attracted the attention of nutritionists for several years. Studies have shown that 8 to 12% of digestible energy is lost as methane in ruminants. Furthermore, methane emissions from the livestock are the major contributor to increase the Greenhouse gases on earth (Johnson and Johnson, 1995). Manipulation of the rumen microbial ecosystem can improve the efficiency of feed utilization and animal performance (Benchaar and Chouinard, 2006). Organic acids, halogen compounds and ionophore antibiotics, in particular, have been shown to decrease the total amount of methane and NH_3-N produced, increasing animal efficiency.

However, concerns regarding the use of antibiotics in animal production have arisen because of awareness of the risk of multidrug-resistant bacteria. The plants have secondary compounds that affect ruminants production positively (Wallace, 2004). These compounds, including phenolic, change the fermentation conditions (pH, propionate proportion, and protein degradation) and therefore affect rumen microbial metabolism (Balcells *et al*. 2012). Studies show that some plant species and their components could be used as alternatives to antibiotics for manipulating rumen fermentation and reducing ruminal methane production, and

improve animal performance (Lin *et al*. 2012).

For example, studies have reported the effects of thyme (Mirzaei-Aghsaghali *et al*. 2011), cinnamon (Jahani-Azizabadi *et al*. 2014), eucalyptus (Joch *et al*. 2016), garlic (Kilic *et al*. 2011) and peppermint (Ozkan *et al*. 2015), on ruminal fermentation patterns. The objective of the present study was to evaluate the effects of dietary addition of herbal plants (alone or in combination) on rumen fermentation pattern (pH, volatile fatty acids, NH_3-N, methane production and digestibility) of diets *in vitro*. Our hypothesis was that the inclusion of herbal plants alone or in combination would have to change the rumen fermentation pattern to reduce methane production in dairy cows that may lead to improved animal production.

MATERIALS AND METHODS

The *in vitro* experiment was carried out at the Faculty of Agriculture of the University of Ferdowsi of Mashhad in Iran. Experiments 1 and 2 were conducted in December 2015 and June 2016, respectively.

Diet and treatments

To evaluate the effect of different HP and HPM, the diet was prepared by taking roughage and concentrate ratio equal to 40:60 (Table 1), milled to pass through 1 mm sieve, and used as the substrate. Two experiments were evaluated in triplicate runs for data analysis using 135 (Exp. 1) and 36 (Exp. 2) bottles (15 and 4 treatments×3 incubation times×3 replications) with a completely randomized design. Set was also incubated devoid of substrate with and without plants that served as blanks for particular treatment; values were corrected for different parameters with these blanks. The physical and chemical composition of the diet used in both experiments as a fermentation substrate *in vitro* incubation is given in Table 1.

Two trials were conducted in this study. In the Exp. 1, different herbal plants were added to the diet at three levels (5, 10 and 15% of concentrate dry matter (DM)): i) garlic (*Allium sativum* L; G_5, G_{10}, G_{15}); ii) eucalyptus (*Eucalyptus globulus* Labill.; E_5, E_{10}, E_{15}); iii) cinnamon (*Cinnamomum cassia* (L.) J.Presl; C_5, C_{10}, C_{15}); iv) thyme (*Thymus vulgaris* L.; T_5, T_{10}, T_{15}); and v) peppermint (*Mentha piperita* (L.) Huds.; P_5, P_{10}, P_{15}). In the Exp. 2, mixture of garlic, cinnamon, eucalyptus, peppermint and thyme were mixed at the percent of 22.22, 11.11, 33.33, 22.22 and 11.11 to make up combinations (HPM). In the second test, a mixture at concentrations of 2, 4 and 6% concentrate DM was used.

Preparation of herbal plant

Garlic (bulb), thyme (stems and leaves), peppermint (stems and leaves) and eucalyptus (leaves) were collected from (FUM) Ferdowsi university of Mashhad's medical herb farm and cinnamon (bark) was purchased.

All of herbs certificated Herbarium of FUM, Research Center for Plant Science, FUM, Mashhad, Iran. Each plant was cut into small pieces and the plant materials were dried at 70 ˚C for 48 h and ground to a final particle size of 1 mm (RetschMuhle mill, Retsch EPP 15×20, Germany).

Chemical analyses of feeds and herbal preparations

Analysis of DM and ash of the herbal plants were measured according to Association Official Analytical Chemists (AOAC, 2005) by oven-drying the samples at 105 ˚C for 48 h and by ashing in a muffle furnace at 550 ˚C for 8 h. Neutral detergent fiber (NDF; assayed without alpha amylase and sodium sulphite) and acid detergent fiber (ADF; assayed without alpha amylase and sodium sulphite) were determined according to Van Soest *et al*. (1991). Ether extract was determined using hexane solvents (AOAC, 2005), and crude protein (CP; N×6.25) was determined using the Kjeldahl (Kjeltec 2300 Autoanalyzer, Foss Tecator AB, Hoganas, Sweden) method (AOAC, 2005). Non-fibre carbohydrates (NFC) were calculated as: 100 − (% NDF+% CP+% ether extract+% ash). The chemical composition of the herbal mixture used in the experiment is given in Table 2.

In vitro incubation

Rumen fluid was collected from three ruminally fistulated dairy cows (600±4.5 kg, body weight) prior to offering the morning feed. Animals were fed 10.4 kg DM, a diet containing alfalfa hay (50%), wheat straw (20%), barley grain (15%), soybean meal (14%) and mineral-vitamin premix (Ca; 195000 mg/kg, P; 90000 mg/kg, Na 55000 mg/kg, Mg; 20000 mg/kg, vit A; 500000 IU, vit D_3 100000 IU and vit E 100 IU) (1%).

Effect of herbal plants on gas production was assessed by incubating approximately 200 mg experimental sample (5, 10 and 15% of concentrate DM) with 30 mL of rumen buffer mixture in 125 mL glass syringes based on Menke and Steingass (1988) procedure. For this procedure, ruminal content was immediately blended and strained through four layers of cheesecloth to eliminate large feed particles, and transferred to the laboratory in a pre-warmed thermos.

A sample of 200 mg was weighed into 125-mL serum bottles, in 3 runs and 9 replicates. The filtrate was then mixed with carbonate buffer (containing ammonium bicarbonate at 4 g/L), then sodium bicarbonate (35 g/L in N-rich incubation medium and sodium bicarbonate at 39.25 g/L in N-low medium), macro-mineral solution (5.7 g anhydrous Na_2HPO_4, 6.2 g anhydrous KH_2PO_4 and 0.6 g $MgSO_4 \cdot 7H_2O$ per liter), and deionized water in a ratio of 1:1:0.5:1.5 and 0.1 mL micro-mineral solution (13.2 g

$CaCl_2 \cdot 2H_2O$, 10.0 g $MnCl_2 \cdot 4H_2O$, 1 g $CoCl_2 \cdot 6H_2O$ and 8.0 g $FeCl_3 \cdot 6H_2O$ per 100 mL) were included. The medium was reduced by the addition of 41.7 mL reducing agent (40 mL deionized water, 1 mL 1 N NaOH and 1 g $Na_2S \cdot 9H_2O$) per liter. Twenty milliliters of medium was dispensed into a 125-mL glass serum bottle, the top of each was stopped with rubber and aluminum caps and placed in a 39 degree centigrade water bath for 96 h. Blank samples, without substrate, were placed throughout the water bath and used to measure gas production from the medium alone.

Rumen liquor was handled under a constant stream of CO_2, all containers used were pre-warmed at 39 degree centigrade and filled with CO_2. Gas production (mL) was recorded at 2, 4, 6, 8, 12, 16, 24, 36, 48, 72 and 96 h. The cumulative gas production data were fitted to the non-linear exponential equation $P= b(1-e^{-ct})$, where b is the volume of gas made from the end of the experiment (mL), c is the fractional rate constant of gas production for b (mL/h), t is the incubation time (h) and P is the volume of gas produced at time t.

Table 1 Feed ingredients and chemical composition of the diet used as substrate in *in vitro* incubation (Exp. 1 and 2)

Item	Control	Herbal plant mixture (HPM)
Ingredient of concentrate (%)		
Corn	30	30
Barley	34	34
Soybean meal	8	8
Cottonseed meal	12	12
Beet pulp	4.8	4.8
Wheat bran	10	10
Calcium carbonate	0.3	0.3
Salt[1]	0.2	0.2
Mineral-vitamin premix[2]	0.2	0.2
Ingredient of roughage (%)		
Alfalfa	15	15
Corn silage	40	40
Wheat straw	45	45
HPM	-	0.60
Chemical composition (g/kg DM)		
Dry matter (g/kg)	880	882
Acid detergent fiber (ADF)	228	229
Neutral detergent fiber (NDF)	483	480
Ether extract (EE)	133.2	136
Ash	77.6	73.5
Crud protein (CP)	131.2	129.6
Organic matter (OM)	922.4	919
Non fiber carbohydrates (NFC)	175	171
Net energy for lactation (NE_L), Mcal/kg DM[3]	1.51	1.50

[1] Contained (per kg): NaCl: 965 g; Zn: 7500 mg; Mn: 5000 mg; Cu: 2500 mg; Fe: 1600 mg; I: 70 mg and Co: 40 mg.
[2] Contained (per kg): Ca: 195000 mg/kg; P: 90000 mg/kg; Na: 55000 mg/kg; Mg: 20000 mg/kg; Vitamin A: 500000 IU; Vitamin D_3: 100000 IU and Vitamin E: 100 IU.
[3] Calculated using values of feed ingredients.

Table 2 Nutritive values analysis of herbal plants

Herbal plant	Composition (%)					% in HPM
	DM	CP	EE	ADF	NDF	
Garlic	94.60	9.21	1.02	9.09	19.48	22.22
Eucalyptus	93.34	5.41	0.90	17.13	31.6	11.11
Cinnamon	94.96	4.50	2.91	34.77	39.54	33.33
Thymus	93.42	6.82	4.12	29.96	39.88	22.22
Peppermint	95.08	12.56	2.92	19.09	31.12	11.11
HPM[2]	94.36	7.05	2.20	24.28	33.33	

DM: dry matter; CP: crude protein; EE: ether extract; ADF: acid detergent fiber; NDF: neutral detergent fiber and HPM: herbal plant mixture.

Methane measurement

For CH_4 measurement, 200 mg of substrate was incubated for 24 h with buffered rumen liquor and respective HP/HPM solution in triplicate. After the stipulated period, total gas production was measured. At the 24 h of the incubation period using the device of multiple gas detector (SR2-BIO System, SEWERIN, Germany). Methane production was determined in three bottles of each treatment in each run.

Volatile fatty acids (VFA), NH_3-N and pH measurement

The supernatant of each syringe including that of blank was used for NH_3-N estimation. Ruminal NH_3-N production was determined by colorimetry similarly to the method of Chaney and Marbach (1962). Briefly, samples were transferred to the laboratory and centrifuged at about 3000 × g for 20 min at 4 ˚C. Exactly 5 mL of supernatants were immediately mixed with 1 mL of HCl 0.2 N and ammonia N was determined by spectrophotometry (Libra S21, Biochrom Technology, Cambridge, UK). For analysis of VFAs, 2 mL of volume supernatants liquid was protected, at –20 ˚C, with 0.5 mL of an acid solution including 20% orthophosphoric acid and 20 m*M* 2-ethylbutyric acid. VFA profile was determined by gas chromatography (YL6100 GC, Young Lin Instrument Co., Anyang, South Korea) equipped with a 50-m (0.32 mm ID) silica-fused column (CP-Wax Chrompack Capillary Column, Varian, Palo Alto, CA, USA) using ethyl-butyric acid as the international standard. The gas flows for helium, hydrogen and air were 10 psi, 30 mL/min and 300 mL/min, respectively. Temperature of injector and detector were 250 and 250 ˚C, respectively. The GC oven temperature program was (1) initial temp 80 ˚C hold 1 min; (2) ramp 20 ˚C/min to 120 ˚C; (3) ramp 6.5 ˚C/min to 140 ˚C hold 7 min and (4) ramp 20 ˚C/min to 205 ˚C. At the end of the incubation period, the pH value was measured in bottles using a portable digital pH-meter with a combination electrode (pH meter 59000-60 pH Tester; Cole-Parmer Instrument Co., Vernon Hills, IL, USA).

***In vitro* digestibility**

At the 24 h of the incubation period, the remaining contents of two bottles were collected for determination of *in vitro* DMD. The DM degradability at 24 h of incubation was calculated as the difference between DM content of substrate before incubation and its undegradable DM after incubation. The metabolizable energy (MJ ME/kg DM), the *in vitro* organic matter digestibility (IVOMD) and short chain fatty acids (SCFA) concentrations of treatments were estimated using the equation of Menke *et al*. (1979) as follows:

ME (MJ/kg DM)= 2.20 + 0.136 GP + 0.057 CP + 0.0029 CP2

IVOMD (%)= 14.88 + 0.889 GP + 0.45 CP + 0.0651 XA

SCFA (mmol per 200 mg DM)= 0.0222 GP – 0.00425

Where:

GP: 24 h net gas production (mL/200 mg DM).

CP: crude protein (%).

XA: ash content (%).

Data analysis

Data were analyzed based on completely randomized design with 3 replications for each level of the plants as treatment. Comparison of the means of non-additive versus HP/HPM groups was tested for significance using Duncan's multiple range test of SAS (2002) using the model:

$$y= \mu + T_i + e_{ij}$$

Where:

y: response variable.

μ: overal mean.

Ti: effect of the herbal plant at each concentration level used.

e_{ij}: residual error.

Mean differences were considered significant at $P < 0.05$.

RESULTS AND DISCUSSION

Experiment 1

The trial results regarding different levels of HP (5, 10 and 15% concentrate DM) on cumulative gas production after 2, 4, 6, 8, 16, 24, 36, 48, 72 and 96 h *in vitro* incubation are presented in Figures 1, 2 and 3, respectively. Data analysis showed significant differences in cumulative GP for different levels of herbal plants for all the time of *in vitro* incubation on.

Total gas production in samples with garlic, eucalyptus, cinnamon, thymus and peppermint were higher when compared to the control. When HP was added at 5 or 10% of the diet, gas production was significantly increased compared to the control ($P<0.01$) and the greatest GP was observed in E_5 and T_5. Total gas production in samples with 15% HP were higher than that of the control (Figure 3) and maximum GP was observed in the C_{15} treatment. Conversely, the P_{15} treatment resulted in the gas production that was decreased compared to the control with increasing doses of HP from 5 to 15%.

The effects of HP on *in vitro* gas production coefficients (b and c), GP and CH_4 are shown in Table 3.

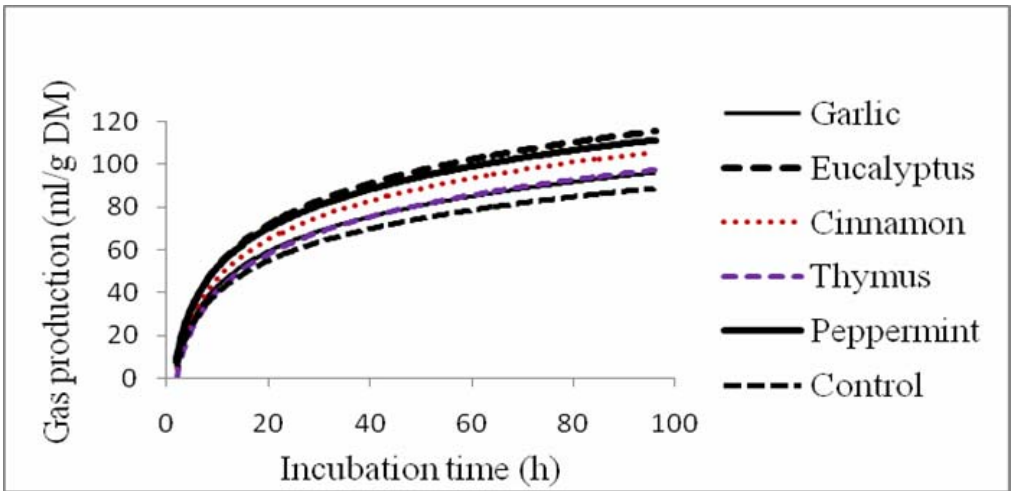

Figure 1 The gas production (mL/g DM) process of level of 5% herbal plant after *in vitro* incubation for 96 h

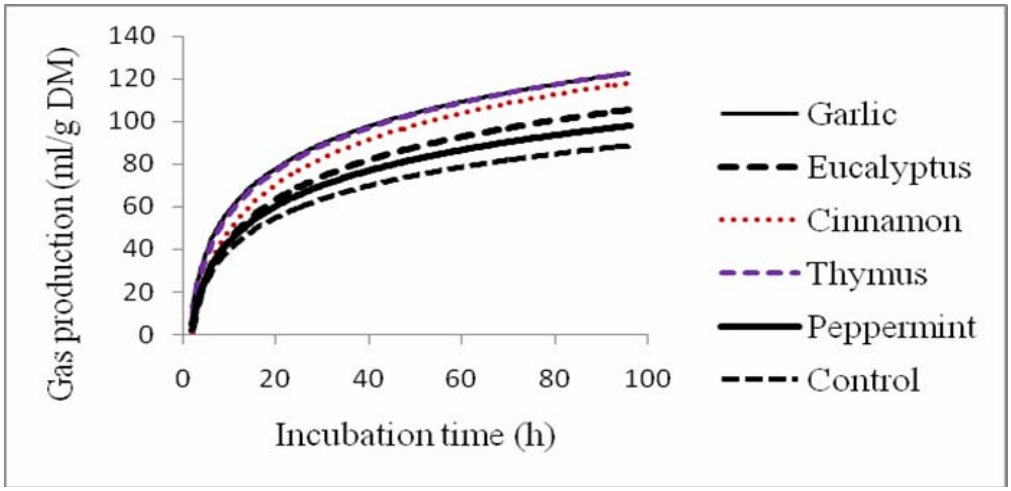

Figure 2 The gas production (mL/g DM) process of level of 10% herbal plant after *in vitro* incubation for 96 h

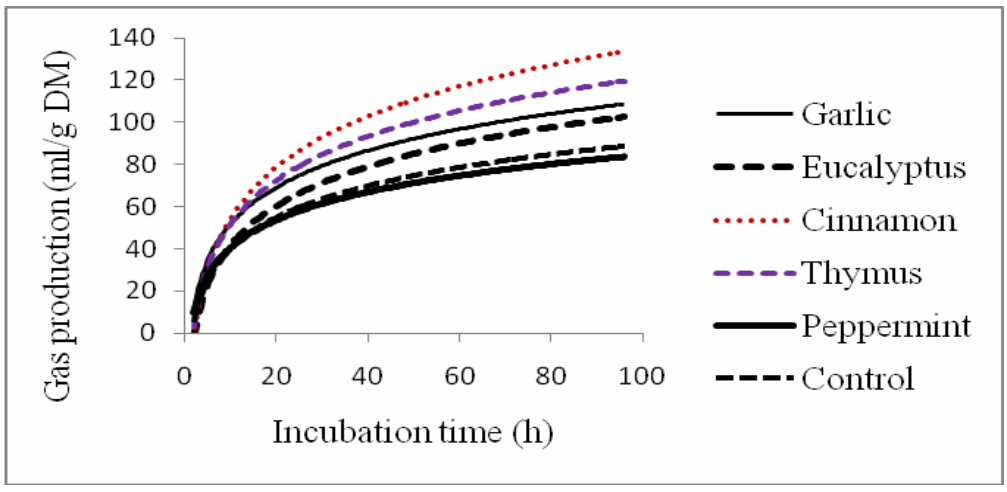

Figure 3 The gas production (mL/g DM) process of level of 15% herbal plant after *in vitro* incubation for 96 h

In accordance with GP, results indicated that gas production coefficients b and c were affected by the inclusion of HP ($P<0.01$).

Among HP used, all concentrations of HP significantly increased the volume of gas produced (b) except P_{15} ($P<0.01$).

Table 3 Effect of herbal plant (HP) source and dosage on gas production (GP) kinetics and cumulative GP after 24 h of *in vitro* incubation

Treatments[1]	Gas production parameters[2]						
	b	c	GP	% change GP	CH_4	% CH_4	% change CH_4
Control	85.5^{igh}	0.051^{cfed}	57.6^{de}	-	53.0^{bc}	22	-
G_5	94.2^{igh}	0.050^{gcfed}	62.5^{bcde}	8.5	36.5^{f}	15	31.81
G_{10}	101.7^{egfh}	0.135^{a}	81.5^{a}	41.4	40.0^{ef}	14	36.36
G_{15}	105.5^{efd}	0.059^{bc}	72.4^{abcd}	25.69	54.4^{bc}	17	22.72
E_5	110.3^{ecd}	0.052^{ced}	77.6^{ab}	34.72	49.1^{dc}	17	22.72
E_{10}	106.3^{efd}	0.039^{h}	63.3^{bcde}	9.8	45.9^{de}	13	40.91
E_{15}	105.9^{efd}	0.037^{h}	77.5^{ab}	34.54	46.1^{de}	13	40.91
C_5	104.9^{egfd}	0.047^{ghfed}	66.1^{abcde}	14.75	47.1^{de}	13	40.91
C_{10}	124.1^{ab}	0.042^{ghfe}	72.1^{abcd}	25.1	37.1^{f}	12	45.46
C_{15}	131.7^{a}	0.041^{ghf}	61.0^{cde}	5.9	50.1^{dc}	14	36.37
T_5	96.1^{igfh}	0.039^{gh}	60.1^{cde}	4.3	45.2^{de}	13	40.91
T_{10}	114.0^{bcd}	0.057^{cbd}	80.5^{a}	39.7	63.7^{a}	20	9.100
T_{15}	119.9^{bc}	0.046^{ghfe}	76.2^{abc}	32.2	44.2^{de}	13	40.91
P_5	105.9^{efd}	0.060^{bc}	72.2^{abcd}	25.3	40.1^{fe}	15	31.81
P_{10}	93.4^{ih}	0.051^{cfed}	61.8^{bcde}	7.2	58.7^{ab}	17	22.72
P_{15}	77.7^{j}	0.064^{b}	55.1^{e}	-4.3	44.6^{de}	13	40.91
SEM	1.01	0.008	1.61	-	1.50	-	-
P-value	0.001	0.01	0.001	-	< 0.01	-	-

[1] Treatments were added to the diet at three levels (5, 10 and 15% concentrate DM). G: garlic; E: eucalyptus; C: cinnamon; T: thymus and P: peppermint.
[2] b: volume of gas produced; c: fractional rate constant of gas production; GP: gas production at 24 h (mL/g DM); CH_4: methane production (mL/g DM) and % CH_4: CH_4 proportion to total gas production.
The means within the same column with at least one common letter, do not have significant difference (P>0.05).
SEM: standard error of the mean.

Moreover, coefficient c was significantly decreased when peppermint was included at 15%, thymus was included at 5% DM and eucalyptus was included at 15% of concentrate DM (P<0.05).

The results showed differences (P<0.05) in cumulative GP after 24 h for the different herb levels. All the herbal plants and concentrations increased GP. Accordingly, gas production was increased from 57.6 to 81.5 mL/g DM from the control to the G_{10}, respectively. The only treatment that did not increase GP was P_{15}, as a decreased by 4.3 mL/g DM was observed.

The methane production, in terms of mL/g DM, during incubation was significantly affected by the HP addition (P<0.01). So that, HP treatments resulted in lower total methane production (CH_4) as compared to the control treatment, with the lowest values (mL/g DM) observed in G_5.

The effects of HP supplementation (5, 10 and 15% of concentrate DM) on *in vitro* ruminal degradability of DMD, OMD, SCFA and ME are shown in Table 4.

The HP affected (P<0.05) the *in vitro* dry and organic matter degradability (DMD and OMD). The G_{10} and C_{15} increased significantly (P<0.05). However, when HP was fed to the diet, the OMD tended to be higher than that of the control. On the other hand, the C_5, G_{10} and C_{15} increased DMD compared to that of the control (P<0.05). The addition of HP in diets led to statistically increase ME (G_{10} and T_{10}) compared to the control, while no significant difference between G_{10} and T_{10} was observed. The supplementation of HP increased SCFA compared to the control (P<0.05).

As shown in Table 5, the concentration of total volatile fatty acids produced during the fermentation with HP was reduced with P_{15} in comparison to the fermentation basal feed (P<0.05) but E_5 treatment resulted in higher VFA concentration than the basal diet (P<0.05). Acetate concentration (mmol/g OMD), propionate concentration (mmol/g OMD), and acetate to propionate ratio (A/P) were affected compared with the control. There were significant differences (P<0.05) in ruminal NH_3-N concentration between animals supplemented with herbs and the control (Table 5).

So that all herb supplements lowered concentrations of NH_3-N (37.15 to 40.45 mg /dL^{-1}) compared to the control treatment (45.05 mg/dL^{-1}). Data analysis showed HP did not affect ruminal pH.

The pH of incubations ranged from 6.52 to 6.73 and from 6.64 to 6.71, respectively. Nevertheless, there was a tendency to increase ruminal pH when T_5 was supplemented (Table 5).

Experiment 2

The trial results regarding different levels of HPM on cumulative gas production after 2, 4, 6, 8, 16, 24, 36, 48, 72 and 96 h *in vitro* incubation are presented in Figure 4 and Table 6. Results indicated that *in vitro* gas production (b) and the fractional rate constant of gas production (c) were affected by the inclusion of HPM (P<0.05).

Table 4 Effect of herbal plant (HP) supplementation (5, 10 and 15% of concentrate DM) on *in vitro* ruminal degradability of DMD, OMD, SCFA and ME

Treatments[1]	Digestibility parameters[2]						
	DMD	OMD		SCFA		ME	
	24 h	16 h	24 h	16 h	24 h	16 h	24 h
Control	43.7^{bc}	49.08^{cd}	57.2^{c}	1.01^{d}	1.33^{c}	9.78^{d}	11.9
G_5	52.8^{abc}	50.34^{cd}	60.2^{bc}	1.13^{cd}	1.38b^{c}	10.51^{cd}	12.1
G_{10}	54.2^{ab}	64.78^{a}	78.4^{a}	1.53^{a}	1.80^{ab}	13.00^{a}	14.5
G_{15}	52.6^{abc}	58.58^{abc}	70.2^{abc}	1.35^{abcd}	1.60^{abc}	11.89^{abcd}	13.3
E_5	46.4^{bc}	57.19^{abcd}	74.8^{ab}	1.37^{abc}	1.71^{abc}	12.03^{abc}	13.9
E_{10}	45.6^{bc}	48.87^{cd}	61.3^{bc}	1.14^{cd}	1.47^{abc}	10.60^{cd}	11.9
E_{15}	46.3^{bc}	45.33^{d}	60.1^{bc}	1.06^{cd}	1.35^{c}	10.13^{cd}	11.8
C_5	55.2^{ab}	51.83^{bcd}	64.6^{abc}	1.18^{bcd}	1.46^{abc}	10.82^{bcd}	12.4
C_{10}	48.9^{bc}	53.22^{abcd}	67.3^{ab}	1.26^{abcd}	1.59^{abc}	11.36^{abcd}	13.3
C_{15}	62.5^{a}	55.56^{abcd}	76.4^{ab}	1.41^{abc}	1.81^{a}	12.25^{abc}	14.6
T_5	50.5^{bc}	47.59^{cd}	59.7^{bc}	1.08^{cd}	1.33^{c}	10.25^{cd}	11.7
T_{10}	45.0^{bc}	63.34^{ab}	72.8^{abc}	1.51^{ab}	1.71^{abc}	12.84^{ab}	13.7
T_{15}	51.3^{abc}	56.10^{abcd}	73.6^{abc}	1.35^{abcd}	1.68^{abc}	11.86^{abcd}	13.8
P_5	45.4^{bc}	58.50^{abc}	70.1^{abc}	1.32^{abcd}	1.59^{abc}	11.69^{abcd}	13.3
P_{10}	43.6^{bc}	50.88^{bcd}	60.8^{bc}	1.12^{cd}	1.36^{c}	10.46^{cd}	14.6
P_{15}	41.1^{c}	53.38^{abcd}	57.3^{c}	1.17^{bcd}	1.3^{c}	10.76^{bcd}	11.3
SEM	1.15	1.10	1.44	0.031	0.03	1.25	0.37
P-value	0.04	0.03	0.03	0.01	0.05	0.01	0.93

[1] Treatments were added to the diet at three levels (5, 10 and 15% concentrate DM). G: garlic; E: eucalyptus; C: cinnamon; T: thymus and P: peppermint.
[2] DMD: dry matter degradability (%); OMD: organic matter degradability (%)= 14.88 + 0.889 GP + 0.45 CP + 0.0651 XA; ME: metabolizable energy (MJ/kg DM)= 2.20 + 0.136 GP + 0.057 CP + 0.0029 CP2; SCFA: short chain fatty acid concentrations (mmol per 200 mg DM)= 0.0222 GP – 0.00425.
The means within the same column with at least one common letter, do not have significant difference (P>0.05).
SEM: standard error of the mean.

Table 5 Changes of the VFAs (mmol/g OMD), NH_3-N (mg/dL^{-1}) and pH by adding different levels of HP after *in vitro* incubation for 24 h

Treatments[1]	Item[2]						pH
	TVFs	Acetate	Propionate	Butyrate	A/P	NH_3-N	
Control	12.18^{bcde}	8.49^{a}	2.73^{e}	0.95	3.12^{a}	45.05^{a}	6.65
G_5	11.86^{de}	7.94^{abcd}	3.066^{de}	0.86	2.59^{b}	41.00^{abcd}	6.60
G_{10}	12.26^{bcd}	8.05^{abc}	3.34^{bcde}	0.87	2.41^{bc}	38.75^{dc}	6.62
G_{15}	12.18^{bcde}	7.66^{bcde}	3.55^{abcd}	0.97	2.16^{cd}	38.50^{dc}	6.63
E_5	13.38^{a}	8.11^{ab}	4.27^{a}	1.00	1.92^{d}	42.05^{abc}	6.61
E_{10}	12.42^{bcd}	7.78^{bcde}	3.82^{abc}	0.82	2.03^{cd}	37.50^{dc}	6.65
E_{15}	12.29^{bcd}	7.51^{cde}	3.86^{abc}	0.92	1.95^{d}	40.45^{bcd}	6.63
C_5	12.32^{bcd}	7.67^{bcde}	3.72^{abcd}	0.91	2.07^{cd}	38.40^{dc}	6.70
C_{10}	12.79^{abcd}	8.03^{abc}	3.69^{abcd}	1.07	2.19^{bcd}	38.60^{dc}	6.59
C_{15}	12.17^{bcde}	7.45^{de}	3.69^{abcd}	1.03	2.02^{cd}	37.90^{dc}	6.59
T_5	12.47^{abcd}	7.90^{bcd}	3.56^{abcd}	1.00	2.21^{bcd}	44.65^{ab}	6.73
T_{10}	12.90^{abc}	8.04^{abc}	3.92^{abc}	0.93	2.06^{cd}	41.45^{abcd}	6.63
T_{15}	12.11^{cde}	7.25^{e}	4.01^{ab}	0.85	1.87^{d}	40.60^{abcd}	6.52
P_5	13.11^{ab}	8.07^{abc}	4.05^{ab}	0.99	1.99^{cd}	39.15^{dc}	6.64
P_{10}	12.73^{abcd}	8.01^{abcd}	3.74^{abcd}	0.97	2.15^{cd}	40.20^{bcd}	6.67
P_{15}	11.30^{e}	7.24^{e}	3.19^{cde}	0.87	2.27^{bcd}	37.15^{d}	6.60
SEM	0.091	0.059	0.071	0.017	0.051	0.47	0.010
P-value	0.003	0.0006	0.002	0.389	0.0001	0.01	0.198

[1] Treatments were added to the diet at three levels (5, 10 and 15% concentrate DM). G: garlic; E: eucalyptus; C: cinnamon; T: thymus and P: peppermint.
[2] TVFs: total volatile fatty acids; A/P: acetate/propionate and NH_3-N: ammonia nitrogen (mg/dL^{-1}).
The means within the same column with at least one common letter, do not have significant difference (P>0.05).
SEM: standard error of the mean.

In vitro gas production (b) was similar in the control, 2% and 4% HPM treatments, while there were differences between 6% HPM and the control (P<0.05).

The addition of 6 percent of HPM significantly decreased (P<0.05) *in vitro* gas production (b).

However, compared with the control, addition of 6% HPM resulted in a 4% increase (P<0.05) in the fractional rate constant of gas production (c). In brief, b and c were increased with HPM supplementation to the diet but CH_4 was decreased with increasing dose of extra HPM (P<0.05).

Table 6 Effect of herbal plant mixture (HPM) on gas production parameters on *in vitro* rumen fermentation of a 60:40 roughage: concentrate diet after incubation for 24 h

HPM	Levels	Gas production parameters[1]							pH
		b	c	GP	% change GP	CH_4	% change CH_4	CH_4/GP	
Control		133.19^{a}	0.050^{b}	89.93^{a}	-	53.53^{a}	-	0.57^{ab}	6.67
HPM	2%	134.67^{a}	0.055^{a}	92.14^{a}	2.57	44.61^{b}	16.66	0.48^{b}	6.54
	4%	134.46^{a}	0.052^{ab}	91.49^{a}	1.84	44.80^{b}	16.30	0.60^{a}	6.65
	6%	127.04^{b}	0.048^{b}	75.47^{b}	-15.98	43.72^{b}	18.32	0.48^{b}	6.71
SEM	-	1.30	0.0009	2.64	-	1.50	-	0.2	0.05
P-value	-	0.05	0.04	0.04	-	0.05	-	0.06	0.43

[1] b: volume of gas produced; c: fractional rate constant of gas production; GP: gas production (mL/g DM) and CH_4: methane production (mL/g DM).
The means within the same column with at least one common letter, do not have significant difference (P>0.05).
SEM: standard error of the mean.

The lowest c was found in 6% treatment and the highest in 2% (see Figure 4). So that, HPM treatments resulted in lower total methane production (CH_4) as compared to the control treatment, with the lowest values (43.72 mL/g DM) observed in 2% HPM (P<0.05).

The effects of different levels of HPM on DMD, OMD, SCFA and ME are presented in Table 7. Data analysis showed that ruminal pH was not affected by levels of HPM and ranged from 6.54 to 6.71. Nevertheless, there was a tendency to decrease ruminal pH when 2% HPM was supplemented.

There were no significant differences in ruminal NH_3-N concentration between animals supplemented with HPM and the control. Notwithstanding concentrations of NH_3-N (39.06 to 44.03 mg/dL^{-1}) tended to be lower for cows fed HPM diet than for those fed the control diet (47.70 mg/dL^{-1}). DMD and SCFA were not affected with none of the levels compared to the control, but the HPM also affected (P<0.05) OMD and ME in 24 h. DMD and ME contents increased non-significantly in 2 and 4% HPM. However, both of these indicators significantly declined by 6 percent increase in HPM (P<0.05).

Table 8 shows the comparison of volatile fatty acids (VFAs) and NH_3-N on HPM treatment. Data analysis showed that VFAs were affected by treatments. The HPM treatments had the effect on total VFA and propionate concentrations (P<0.05) and no effect on acetate, but butyrate and acetate to propionate ratio tended to be decreased (P=0.07 and P=0.09, respectively) by HPM. The addition of either 2% HPM or 6% HPM with substrate resulted in higher TVFs (P=0.02) and propionate (P=0.0002) in comparison to the control.

There is very little information available on plants or their major compounds on microbial activity in the rumen of dairy cows. In Exp. 1 and 2, the addition of HP and HPM to the diet significantly increased total *in vitro* gas production (b) and the fractional rate constant of gas production (c, P<0.01). All levels of HP, with the exception of the P_{15} treatment, significantly increased gas production compared to the control.

This is similar to the observations of Menke and Steingass (1988), who also reported an effect of herbal plant on total gas production, and gas production parameters (b and c). It has been strongly suggested that the amount of gas production is positively correlated with diet digestibility (Makkar, 2002). Therefore, increasing the amount of gas production at increased concentrations of herbal plants may represent improved digestibility of feedstuffs. The significant decline in the volume of gas produced in 15% treatments potentially indicates a negative effect on microbial fermentation at that concentration. Similar to our study, Joch *et al.* (2016) observed that *in vitro* GP increased significantly with plant extracts supplementation (including: eugenol, carvacrol, citral, limonene, 1,4-cineole, p-cymene, linalool, bornyl acetate, α-pinene, and β-pinene). They noted that the increase of gas volume might be due to the inclusion of soluble sugars in the reaction mixture. Similarly, Tag El-Din *et al.* (2012) conducted a trial to determine the effect of garlic on gas production, organic matter digestibility and methane emission *in vitro*.

It was concluded that the supplement of garlic increased GP and reduced CH_4 production. In Exp. 2, as compared to the control, the addition of 6% HPM decreased (P<0.05) GP in the present study. Similar to our study, Blancha *et al.* (2016) observed that *in vitro* GP decreased significantly with herbal and plant extracts supplementation in a dose-dependent manner. Gas production in the rumen can be built in two ways: a sum of direct GP because of fermentation (CO_2 and methane) and the GP from buffering of VFA (Kamalak *et al.* 2011). The significant decline in the volume of gas produced in 6% treatment potentially indicates a negative effect on microbial fermentation at that concentration (Cobellis *et al.* 2016). The main antimicrobial effect of herbal plants and essential oil (EO) in the rumen could be attributed to the presence of terpenoids and phenylpropanoids. The effects were related to their chemical structure in that they may aggregate in the cell membrane. Its hydrogen-bonding ability and its proton-release ability may induce conformational modification of the membrane resulting in the cell death (Hundal *et al.* 2016).

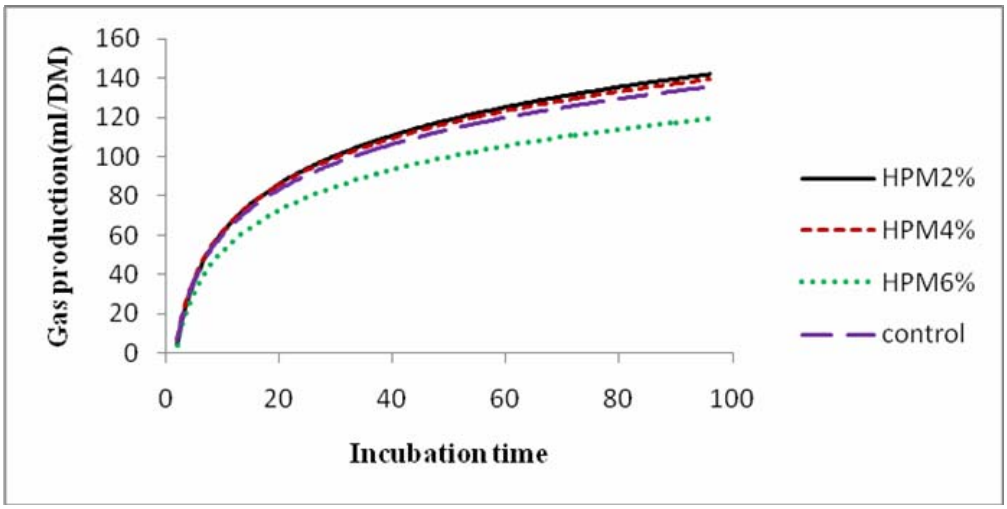

Figure 4 Effect of herbal plant mixture on gas production (mL/DM) after *in vitro* incubation for 96 h

Compared with the control, the addition of HP and HPM into the basal diet resulted in a decrease of methane production ($P<0.05$).

These findings are consistent with prior results of different types of herbal plant supplementation to diets that showed the addition of herbal plant and its component into the basal diet resulted in a decrease of methane production (Dong *et al*. 2010). It seems the decreasing ruminal methane output could improve animal efficiency due to reduced losses of feed energy.

However, the true mechanisms of this effect remain unknown, but the reduction in methane may be due to the inhibition of fiber degradation and decline in methanogenic archaea. The trace compounds in the herbal plants may inhibit methanogenic archaea and or selectively affect microbial fermentation, subsequently decreasing methanogenesis. Additionally, a decrease in methane production may have occurred because of a reduction in the number of rumen protozoa (Ozturk *et al*. 2012).

In Exp. 1, the inclusion of HP increased the DMD and SCFA production of diet. These findings are consistent with Nanona *et al*. (2014), who demonstrated that various doses and different sources of plant tannins have varying effects on DM digestibility *in vitro*. However, it is believed that most active compounds in plants do not affect SCFA concentration and DMD. In Exp. 2, the OMD and ME were lower in 6% HPM compared to the control, but the DMD and SCFA were not influenced by all concentrations of HPM. Although, such result is not in agreement with the finding of Yang *et al*. (2010), but these results are in agreement with findings of Ozkan *et al*. (2015) who suggested that supplementation of EO decreased the OMD, and ME.

These effects could be linked to the antibacterial attributes of herbal plants and EO (containing depression of the cell wall, damage to the cytoplasmatic membrane, leakage of cell contents and coagulation of cytoplasm) that decreased the activity of the bacteria in the rumen (Burt, 2004).

In the previous studies, NH_3-N concentration has been shown to decrease with the addition of herbal plants (Jahani-Azizabadi *et al*. 2014). The inclusion of a specific blend of essential oil compounds caused a decline in NH_3-producing bacteria and a decreased rate of NH_3 production. Consequently, this indicates that the active ingredient in the plants may decrease ruminal proteolysis and amino acid degradation (Molero *et al*. 2004). Wallace *et al*. (2002) found that the major mechanism of function of herbal active ingredients was the limitation of selectively target bacterial adhesion to feed particles in the rumen, and thus, amino acid deamination and NH_3 production were reduced. These effects primarily occur through interference specifically with hyper-NH_3-producing bacteria. The decline in NH_3-N concentration with the addition of thyme essential oil occurred by preventing the breakdown of proteins or peptides into amino acids, and through inhibition of the deamination reaction and or maybe there is an increase in N fixation by specific carbohydrate-degrading bacteria (e.g. cellulolytic organisms).

McIntosh *et al*. (2003) noted that a mix of EO, including thymol reduced the rate of amino acid deamination and prevented the growth of a specific group of ammonia producing bacteria. The prevention of amino acid deamination has practical implications, like potentially altering site of protein digestion from the rumen to the intestine (Patra and Yu, 2012).

Table 7 Effect of herbal plant mixture (HPM)[1] on *in vitro* digestibility of a 60:40 roughage: concentrate diet after incubation for 16, 24 and 48 h

Treatments	Levels	Digestibility parameters[2]									
		DMD		OMD		SCFA			ME		
		24 h	16 h	24 h	48 h	16 h	24 h	48 h	16 h	24 h	48 h
Control	-	49.26	42.90	55.43^{a}	51.56	1.71	1.44	2.71	13.92	15.70^{a}	66.87^{a}
HPM	%2	50.93	46.00	58.06^{a}	59.00	1.71	1.48	2.70	13.88	16.01^{a}	70.04^{a}
	%4	49.33	43.63	56.48^{a}	59.80	1.38	1.47	2.29	11.89	15.92^{a}	68.13^{a}
	%6	47.96	45.26	49.48^{b}	60.83	1.70	1.42	2.59	13.87	13.74^{b}	59.70^{b}
SEM	-	0.80	2.33	1.03	2.89	0.05	0.1	0.07	0.35	0.35	1.24
P-value	-	0.69	0.77	0.003	0.58	0.09	0.13	0.10	0.09	0.04	0.0003

[1] HPM were added to the diet at three levels (2, 4 and 6% concentrate DM).
[2] DMD: dry matter degradability (%); OMD: organic matter degradability (%)= 14.88 + 0.889 GP + 0.45 CP + 0.0651 XA; ME: metabolizable energy (MJ/kg DM)= 2.20 + 0.136 GP + 0.057 CP + 0.0029 CP2; SCFA: short chain fatty acid concentrations (mmol per 200 mg DM)= 0.0222 GP – 0.00425.
The means within the same column with at least one common letter, do not have significant difference (P>0.05).
SEM: standard error of the mean.

Table 8 Changes of the VFAs (mmol/g OMD), acetate/propionate ratio and pH by adding different levels of HP and HPM after *in vitro* incubation for 24 h

Treatments[1]	Levels	Item[2]						pH
		TVFs	Acetate	Propionate	Butyrate	A/P	NH_3-N	
Control	-	11.40^{b}	7.66	2.73^{c}	1.00	2.8	47.70	6.67
HPM	%2	12.83^{a}	7.71	4.19^{a}	0.89	1.84	44.03	6.54
	%4	12.95^{a}	7.85	4.1^{a}	1.00	1.91	41.83	6.65
	%6	12.16^{ab}	7.59	3.49^{b}	1.07	2.18	39.06	6.71
SEM	-	0.22	0.11	0.18	0.02	0.13	2.71	0.05
P-value	-	0.02	0.91	0.0002	0.07	0.09	0.51	0.43

[1] HPM were added to the diet at three levels (2, 4 and 6% concentrate DM).
[2] TVFs: total volatile fatty acids; A/P: acetate/propionate and NH_3-N: ammonia nitrogen (mg/dL^{-1}).
The means within the same column with at least one common letter, do not have significant difference (P>0.05).
SEM: standard error of the mean.

Castillejos *et al*. (2007) showed that supplementation of thymol to rumen liquid caused a reduction in the NH_3-N and an accumulation of amino acids nitrogen. Wallace *et al*. (2002) expressed that amount of ammonia-nitrogen production from amino acids in rumen liquid was by dietary herbals. Feeding herbal plants reduced the numbers of protozoa, which appears to be mediated by the active compounds in these plants (Valdez *et al*. 1986).

The results showed that ruminal pH was not affected by HP and HPM supplement. Similarly, Castillejos *et al*. (2007) also reported that plant essential oil mixtures did not affect rumen pH. Although previous studies have offered mixed reports**,** it seems that these differing results may be partially explained by the experimental conditions, including the type of diets used in the experiments, plant species and/or their active composition used, as well as the rumen pH.

Calsamiglia *et al*. (2007) observed that effects of plant EO are associated with pH and diet, and their use could be beneficial under specific conditions. Plants contain one or more predominant active molecules (secondary metabolites), which are responsible for specific biological effects. In our results, HP increased concentrations of fatty acids and lowered the A/P ratio.

Low A/P ratio indicates more rapid rumen fermentation, indicating the potential for greater energetic efficiency by supplementing with HP.

Acetate to propionate ratio can be an indirect indicator of methane production because propionate can consume free hydrogen that would otherwise be used for methanogenesis by methanogenic archaea (Van Nevel and Demeyer, 1979). Some research has shown that herbal plants increased the total number of bacteria due to a reduction in protozoa predation (Wang *et al*. 2000).

CONCLUSION

In the present study supplementary incubation of herbal plants and its extracts had improved rumen fermentation and decreased methane production that may lead to improved animal production. Herbal plants may provide a natural alternative to synthetic rumen modifiers, which may lead to greater consumer acceptability of these products compared to currently available antibiotic feed additives.

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