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Evaluating Effects of Natural Plant Extract on Ruminal Fermentation Using *in vitro* Gas Production Technique

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Abstract: The aim of this study was to determine effects of the increasing concentrations of thyme methanolic extract (0 and 0.3 ml/30 ml buffered rumen fluid) on Soybean Meal (SBM) degradability were studied by *in vitro* gas producing techniques. Fermentation of treatment samples were carried out with rumen fluids obtained from three mature cannulated steers in times of 2, 4, 6, 8, 12, 24, 48, 72 and 96 h were performed. The results showed that gas volume at 72 h incubation time (for 200 mg dry samples), of soybean meal and thyme methanolic extract (0.3 ml/30 ml buffered rumen fluid) were 62.91 and 37.64 (ml/200 mg DM) respectively. The gas production from soluble fraction (a), the gas production from insoluble fraction (b), rate constant of gas production during incubation (c) and the potential gas production (a+b) contents of soybean meal were 4.42 (ml/200 mg DM), 67.1 (ml/200 mg DM), 0.113 (ml/h) and 71.52 (ml/200 mg DM), while for thyme methanolic extract (0.3 ml/30 ml buffered rumen fluid) were 1.006 (ml/200 mg DM), 42.8 (ml/200 mg DM), 0.033 (ml/h) and 43.81 (ml/200 mg DM) respectively.

Key words: Thyme methanolic extract, soybean meal, gas production technique, incubation, potential gas production, fermentation

INTRODUCTION

The continuing increase in world population escalates demand for food, among which proteins are extremely important (Bunthoeun *et al*., 2007). Some protein is formed in plants, fishes, mammals, but the meat and milks of grazing animals have long been an important source of protein and among these animals the ruminants (cattle, sheep and goats) predominate (Hungate, 1988; Bunthoeun *et al*., 2007). Furthermore, ruminants play an important role in the livelihood of farmers in the developing world, providing sustenance as milk and meat, animal traction, manure for crop production, cash income from sales of their products and a safety net of capital assets to face risks and misfortune in harsh environments (Orskov, 1993; Bunthoeun *et al*., 2007). In ruminants, nutrient inputs are first to fermentative digestion by ruminal microorganisms. The microbial fermentation products eventually become available as energy (volatile fatty acids) and protein (microbial cells) for animal tissue metabolism. However, ruminal fermentation also produces methane (CH_4), carbon dioxide (CO_2) and ammonia (NH_3). Methane production in the rumen is an energetically wasteful process, since the portion of the animal's feed, which is converted to CH_4, is eructated as gas. Furthermore, emissions of CH_4 into the environment contribute to global warming (Johnson and Johnson, 1995; Bunthoeun *et al*., 2007) by trapping outgoing terrestrial infrared radiation 20 times more effectively than CO_2, which leads to increased surface temperatures and indirectly affects atmospheric oxidation reactions that produce CO_2 (Bunthoeun *et al*., 2007). In addition, emissions of NH_3, nitrous oxide (N_2O) and CH_4 from ruminant manures and urine to the environment affect water quality and human health and also contribute to greenhouse gases. Emissions of N have been associated with adverse effects on human health (Knobeloch *et al*., 2000; Bunthoeun *et al*., 2007) such as bronchitis and asthma attacks (McCubbin *et al*., 2002; Bunthoeun *et al*., 2007). Nitrogen compounds in water contribute to water quality problems because of excess algae growth in streams, lakes, reservoirs and coastal waterways. Thus, there is increased worldwide interest in addressing mitigation of CH_4 in animal agriculture and to reduce N loss in manure and urine (Bunthoeun *et al*., 2007). In order to delay ruminal protein degradation, dietary protein was denatured by treatment with formaldehyde or more controversially, antibiotics were used to suppress the bacterial populations responsible for the rapid protein fermentation. But the use of such compounds has been criticized, as they may leaves harmful residues in the food chain and promote the spreading of resistance genes. These led to its prohibition in European Union in 2006 in animal feeding. Accordingly there is greater interest in using plants and plant extract as alternatives to feed antibiotics to manipulate ruminal fermentation, improve feed efficiency and animal productivity

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(Benchaar *et al*., 2007; Patra *et al*., 2006). *In vitro* gas production technique provide cost effective, easy to determine and suitable for use in developing countries. This method also can predicts feed intake (Khazaal *et al*., 1995; Mirzaei-Aghsaghali *et al*., 2011), digestibility, microbial nitrogen supply, amount of short chain fatty acids, carbon dioxides and metabolizable energy of feeds for ruminants (Menke and Steingass, 1988; Babayemi, 2007; Mirzaei-Aghsaghali *et al*., 2008b, Mirzaei-Aghsaghali and Maheri-Sis, 2008a; Maheri-Sis *et al*., 2008, 2007). The cumulative volume of gas production increased with increasing time of incubation. Although there are other models available to describe the kinetics of gas production, the (Orskov and McDonald, 1979; Mirzaei-Aghsaghali and Maheri-Sis, 2008a, Mirzaei-Aghsaghali *et al*., 2008b; Maheri-Sis *et al*., 2007, 2008) was chosen because the relationship of its parameters with intake, digestibility and degradation characteristic of forages and concentrate feedstuffs had been documented (Maheri-Sis *et al*., 2011). The objective of this study was to evaluate the potential of natural plant extract on fermentation pattern by the *in vitro* gas production technique.

MATERIALS AND METHODS

Procedure of thyme extracts preparation: The thyme extracts were prepared according to Patra *et al*. (2006) with some modifications. The thyme materials were dried at 50°C and ground in mills to pass a 1 mm sieve and 100 g placed in 1000 ml of methanol solvent (%95). The bottle of all the solvents were stoppered and agitated with a magnetic stirrer for 24 h at room temperature. Then the solutions were filtered through Whatman 1 filter paper. The residue was re-extracted with 500 ml of methanol for 24 h staying at room temperature and be filtered through Whatman 1 filter paper. Extracts were combined. Methanol was evaporated from the solution at approximately 45°C by using a rotary evaporator. The concentrated extract was stored at 4°C for further use.

Treatments and experimental design: The different levels of thyme extracts were added to the diet sample (soybean meal). Two levels (0 and 0.3 ml/30 ml buffered rumen fluid) of plant extract in a syringe (100 ml) containing 200 mg of milled (1mm) soybean meals were investigated as follow 1-no additive 2-thyme extracts (0.3 ml/30 ml buffered rumen fluid).

***In vitro* gas production:** *In vitro* gas production measurements were carried out in the laboratory of Animal Science Research Institute in Karaj. Fermentation of Soybean Meal (SBM) samples were carried out with rumen fluid obtained from three mature cannulated steers (age = 4.5 to 5 years; BW = 416 kg) fed twice daily with a diet (DMI = 8 kg/day) containing 70% hay (dry alfalfa and wheat straw with 70 to 30 ratio) and 30% concentrate (35% barley meal, 17% soybean meal, 25% whole cottonseed, 20% wheat bran, 1% $CaCO_3$ and 2% minerals and vitamins) as total mixed ratio, following the method described by Menke and Steingass (1988). Water was available *ad libitum*. The inoculum was prepared as described by Menke and Steingass (1988). It consisted of the rumen liquor mixed (1:2 v/v) with anaerobic artificial saliva. The latter included, for a final volume of 1 L, 237 ml of buffer solution, 237 ml of a main element solution, 0.12 ml of a trace element solution, 1.22 ml of resazurin solution (100 mg resazurin made up to 100 ml distilled water), 49.5 ml of a reduction solution (prepared fresh and separately and consisting of 2 ml of NaOH 1 N, 285 mg of Na_2S-$7H_2O$ and 47.5 ml distilled water for 1 L saliva), completed with 475 ml of distilled water. The buffer solution consisted of $NaHCO_3$ 35 g/l and NH_4HCO_3 4 gl. The main element solution consisted of Na_2HPO_4 5.70 g/l, KH_2PO_4 6.20 g/l and Mg SO_4-$7H_2O$ 0.60 g/l. The trace element solution consisted of $CaCl_2$-$2H_2O$ 13.20 g, $MnCl_2$-$4H_2O$ 10.00 g, $CoCl_2$-$6H_2O$ 1.00 g and $FeCl_2$-$6H_2O$ 0.80 g made up to 100 ml with distilled water. The rumen liquor was incorporated in the medium once the reduction process was achieved (resazurin decoloration after adding the reduction solution). All manipulations were done under continuous CO_2 reflux. Approximately, 200 mg Soybean Meal (SBM) ground samples were weighed into the glass syringes of 100 ml. The fluid-buffer mixture (30 ml) was transferred into the glass syringes of 100 ml. The glass syringes containing Soybean Meal (SBM) samples and rumen fluid-buffer mixture were incubated at 39°C. The syringes were gently shaken 30 min after the start of incubation. The gas production was determined at 2, 4, 6, 8, 12, 24, 48, 72 and 96 h of incubation. All samples were incubated in triplicate with three syringes containing only rumen fluid-buffer mixture (blank). The net gas productions for Soybean Meal (SBM) samples were determined by subtracting the volume of gas produced in the blanks (Menke and Steingass, 1988). Gas production data were fitted to the model of Orskov and McDonald (1979).

$$Y = a + b\ (1\text{-}e\text{-}ct) \quad (1)$$

Where, (a) is the gas production from the immediate soluble fraction (ml); b is the gas production from the immediately insoluble fraction (ml); c is the gas production rate constant for the insoluble fraction ($ml.h^{-1}$); a + b is the potential gas production (ml); t is the incubation time (h) and Y is the gas production at time t.

Statistical analysis: Data on apparent gas production parameters were subjected to one-way analysis of variance using the analysis of variation model ANOVA of SAS (2000). Multiple comparison tests used Duncan's

multiple-t-test (1980). Significance between individual means was identified using the Duncan's multiple range tests. Mean differences were considered significant at ($p<0.0001$). Standard errors of means were calculated from the residual mean square in the analysis of variance. All data obtained from three replicates n = 3.

RESULTS AND DISCUSSION

Gas production volumes (ml/200mg DM) at different incubation times shown were in Fig. 1 and 2.

Gas production volumes (ml 200 mg DM) at different incubation times of soybean meal and thyme methanolic extract (0.3 ml/30 ml buffered rumen fluid) are presented in Table 1.

The gas volumes for soybean meal in different incubation times were higher than that of thyme methanolic extract (0.3 ml/30 ml buffered rumen fluid) ($p<0.0001$). The results showed that gas volume at 2 hours incubation time (for 200 mg dry samples), were 9.31 and 3.64 ml/200 mg DM for soybean meal (control) and thyme methanolic extract (0.3 ml/30 ml buffered rumen fluid), respectively. The results showed that gas volume at 4 h incubation time (for 200 mg dry samples), were 19.96 and 5.81 ml/200 mg DM for soybean meal (control) and thyme methanolic extract (0.3 ml/30 ml buffered rumen fluid), respectively. The results showed that gas volume at 8 hours incubation time (for 200 mg dry samples), were 37.26 and 7.13 ml/200 mg DM for soybean meal (control) and thyme methanolic extract (0.3 ml/30 ml buffered rumen fluid), respectively. Gas volume at 12 h incubation time (for 200 mg dry samples), of Soybean Meal (SBM) and thyme methanolic extract (0.3 ml/30 ml buffered rumen fluid) were 46.24 and 11.28 (ml/200 mg DM), respectively. Gas volume at 24 h incubation time (for 200 mg dry samples), of soybean meal and thyme methanolic extract (0.3 ml/30 ml buffered rumen fluid) were 56.38 and 23.71 (ml/200 mg DM) respectively. Gas volume at 48 h incubation time (for 200 mg dry samples), of soybean meal and thyme extract 0.3 were 62.12 and 35.9 (ml/200 mg DM) respectively. Gas volume at 72 h incubation time (for 200 mg dry samples), of soybean meal and thyme methanolic extract (0.3 ml/30 ml buffered rumen fluid) were 62.91 and 37.64 (ml/200 mg DM) respectively.

Estimated parameters of Soybean Meal (SBM) and thyme methanolic extract (0.3 ml/30 ml buffered rumen fluid) are presented in Table 2.

The gas production from soluble fraction (a), the gas production from insoluble fraction (b), rate constant of gas production during incubation (c) and the potential gas production (a + b) contents of soybean meal were 4.42 (ml/200 mg DM), 67.1 (ml/200 mg DM), 0.113 (ml/h) and 71.52 (ml/200 mg DM), respectively. The gas

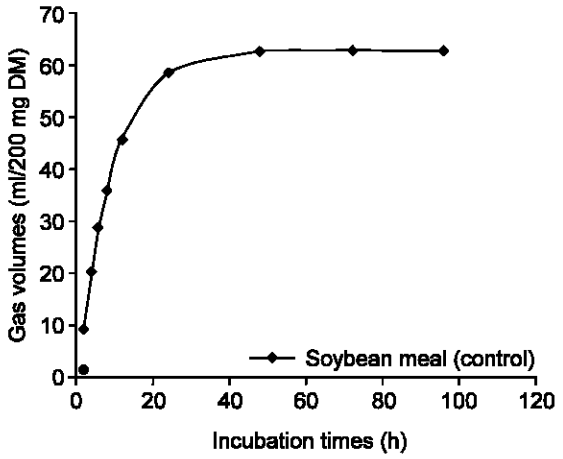

Fig. 1: *In vitro* gas production volume of soybean meal was different incubation time

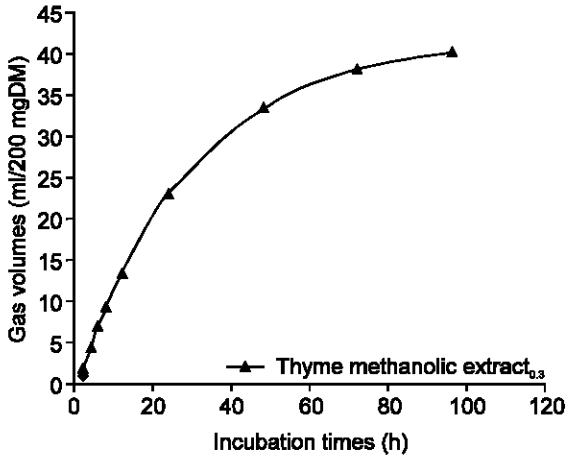

Fig. 2: *In vitro* gas production volume of thyme methanolic extract (0.3 ml/30 ml buffered rumen fluid) was different incubation time

production from soluble fraction (a), the gas production from insoluble fraction (b), rate constant of gas production during incubation (c) and the potential gas production (a + b) contents for thyme methanolic extract (0.3 ml/30 ml buffered rumen fluid) were 1.006 (ml/200 mg DM), 42.8 (ml/200 mg DM), 0.033 (ml/h) and 43.81 (ml/200 mg DM), respectively. Borchers (1965) was the first to report the potential benefit of essential oils on rumen microbial fermentation. Borchers observed that the addition of thymol (active compound of thyme and oregano) to rumen fluid *in vitro* resulted in the accumulation of ammonia acid and the reduction of ammonia nitrogen concentrations (Calsamiglia *et al*., 2007). Thymol (active compound of thyme) is a monoterpene [5-methyl-2-(1methylethyl) phenol; C10H14O] with strong antimicrobial activity against a wide range of gram-positive and negative bacteria

Table 1: Gas production volume (ml/200 mg DM) of soybean meal and thyme methanolic extract (0.3 ml/30 ml buffered rumen fluid) at different incubation times

	Treatments	
Time (h)	No additive (control)	Thyme extract$_{0.3}$
2	9.31	3.64
4	19.96	5.81
6	27.62	6.30
8	37.26	7.13
12	46.24	11.28
24	56.38	23.71
48	62.12	35.90
72	62.91	37.64
96	63.72	39.06

Table 2: Estimated parameters gas production of soybean meal and thyme methanolic extract (0.3 ml/30 ml buffered rumen fluid)

	Control	Thyme extract$_{0.3}$
a (ml)	4.420	1.006
b (ml)	67.100	42.800
(a + b) (ml)	71.520	43.810
c (ml/h)	0.113	0.033

a: The gas production from soluble fraction (ml/200 mg DM), b: The gas production from insoluble fraction (ml/200 mg DM), c: Rate constant of gas production during incubation (ml/h), (a + b): The potential gas production (ml/200 mg DM)

(Burt, 2004; Calsamiglia *et al*., 2007, 2006). Salamatazar *et al*. (2011) estimation effect of tree doses thyme methanolic extract (0, 0.15 and 0.3 ml/30 ml buffered rumen fluid) on degradability kinetics, of sunflower meal and report gas volume at 48 h incubation (for 200 mg dry samples), soluble fraction (a), insoluble but fermentable fraction (b), potential gas production (a + b) and rate constant of gas production (c) of sunflower meal were 44.99, 3.60, 49.32, 52.92 ml/200 mg DM and 0.135 ml/h, gas volume at 48 h incubation (for 200 mg dry samples), soluble fraction (a), insoluble but fermentable fraction (b), potential gas production (a + b) and rate constant of gas production (c) of thyme methanolic extract (0.15 ml/30 ml buffered rumen fluid) were 29.91, 0.53, 36.25, 36.79 ml/200 mg DM and 0.049 ml/h, respectively. Rezaei *et al*. (2011) evaluation effect of tree doses clove methanolic extract (0, 0.5 and 1 ml/30 ml buffered rumen fluid) on degradability, of soybean meal and report gas volume at 48 h incubation (for 200 mg dry samples), soluble fraction (a), insoluble but fermentable fraction (b), potential gas production (a + b) and rate constant of gas production (c) of soybean meal were 71.240, 1.767, 70.880, 72.647 ml/200 mg DM and 0.100 ml/h, gas volume at 48 h incubation (for 200 mg dry samples), soluble fraction (a), insoluble but fermentable fraction (b), potential gas production (a+b) and rate constant of gas production (c) of clove methanolic extract (1 ml/30 ml buffered rumen fluid) were 22.717, 8.914, 19.516, 28.429 ml/200 mg DM and 0.051 ml/h, respectively. Gas volume at 72 and 96h incubation (for 200 mg dry samples), of soybean meal were 72.24 and 74.360 ml/200 mg DM, while for clove methanolic extract (1 ml/30ml buffered rumen fluid) were 25.383 and 29.130 ml/200 mg DM, respectively. The gas production from soluble fraction (a) and the potential gas production (a + b) contents in this study were similar to the results of Salamatazar *et al*. (2011), while The gas production from soluble fraction (a) and the potential gas production (a + b) content was lower than those reported by Rezaei *et al*. (2011).

Conclusion: This study the gas volumes for soybean meal in different incubation times were higher than that of thyme methanolic extract (0.3 ml/30 ml buffered rumen fluid) ($p<0.0001$), suggested that the doses 0.3 thyme methanolic extract have the potential to affect ruminal fermentation efficiency and be a promising methane mitigating agent.

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