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Evaluation Effects of Clove Methanol Extract on Methane Production in the *in vitro* Condition

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Abstract: The aim of this study was to investigate effects of different levels of Clove Methanol Extract (CME) on methane production in ruminant with practical diets. Two experimental diets (diet1: 100% forage and diet 2: 100% barley) were used to investigate effects of 3 levels of CME (0, 0.5 and 1 ml) on methane production at 2, 4, 12, 16, 24, 48, 72 and 96 h of incubation. Gas production was continuously measured by EX-TEC HS 680 GC. Cumulative gas production was recorded at 2, 4, 12, 16, 24, 48, 72 and 96 h of incubation periods. Data processed in excel and analyzed using SAS statistical analyze software. Result show that Clove methanol extract in initial time of incubation (2 and 4 h) not affects on the decreased of methane production, but increase incubation time CME significantly decreased methane gas volume in two diets and all of CME levels and could significantly decrease methane volume in the treatment. According to result we could suggest CME for improve methane production in the *in vitro* condition and need further research for application CME in field of ruminant nutrition.

Key words: Methanol extract, clove, methane production

INTRODUCTION

The gas production method is also utilized to study feed additives and rumen fermentation modifiers, such as ether extract, by incubating feeds in the presence or absence of these compounds. Rumen modifiers are compounds that are added to the diet to modify the populations of bacteria in the rumen. For example, some compounds are fed to reduce methanogenic bacteria to reduce methane production in the rumen. Manipulations of rumen microbial ecosystem for reducing methane emission by ruminants to improve their performance are some of the most important goals for animal nutritionists. One of the powerful greenhouse gases contributing global warming is methane. Johnson and Johnson (1995) reported methane production represents not only an energetic loss for the animal but also contributes to the global warming because methane is released to the atmosphere. Methane production in the domain of Archaea is characterized by their ability to produce methane in anoxic conditions by Guo *et al.* (2005). Among the different sources of methane emission such as wetlands, temporal fields, energy sectors, ruminants, landfills and biomass burning, Wright *et al.* (2004) and Mastepanov *et al.* (2008) reported that methane from enteric fermentation constitutes the largest source. Mitigation of methane may contribute to lowering the greenhouse effect that is a topical issue for the scientific community. However, there is limit information available concerning the

composition and numbers of methanogens and the effect of variations in diet on these populations by Kumar *et al.* (2009).

FAO researchers (2003) believe that if methane emissions continue to rise in direct proportion to livestock number a 60% increase in global methane production is predicted by 2030. However, changes in feeding strategy could remodel the present scenario of methane emission from livestock and thus mitigate some of this increase. According to the US-EPA (2006) pooled methane emission from enteric fermentation and manure management attributable to domesticated livestock are predicted to increase by 21% from 2005-2020. Possible changes in animal production practices for reducing methane emission are discussed below.

To decrease the methane production from ruminants, feeding strategies need to be studied. One of the strategies is feed additives. Moreover, various feed supplements have been found to directly or indirectly reduce methane emissions, including halogenated methane analogues by Ungerfeld *et al.* (2004). One of the feed additives is ether extract. Plant extracts with high concentration of secondary metabolites are good candidates for achieving one or more of these objectives by Teferedegne (2000). Clove bud oils have biological activities, such as antibacterial, antifungal, insecticidal and antioxidant properties and are used traditionally as flavoring agent and antimicrobial material in food by Huang *et al.* (2002). Baker (1999) reported the

methanogens are a large and highly diverse group of Archaea and according to Boone *et al.* (2001) comprising 5 genera and 23 species isolated in pure culture. These are present in diverse natural anaerobic ecosystems e.g. wetlands, marshes, activated sludge, rhizosphere, rumen etc. and obtain their energy from the reduction of carbon dioxide, formate, the methyl moiety of methanol, acetate and some methyl amines by Jones (1991). Thymol and clove or fennel extracts have also reduced methane production but also reduced digestibility or propionate concentration by Patra *et al.* (2005a,b). Eugenol is a phenolic compound with wide-spectrum antimicrobial activity against gram-positive and gram-negative bacteria and it is one of the main active components in clove bud (*Eugenia caryophyllus* or *S. aromaticum*) and cinnamon (*C. cassia*) oils. Clove bud oil also affected Nitrogen (N) metabolism, increasing peptide N and numerically decreasing Amino acid N concentrations, suggesting that it decreased the peptidolytic activity in the rumen by Calsamiglia *et al.* (2007). According to Busquet *et al.* (2006) in an *in vitro* batch culture dose-response study, reported that clove bud oil affected rumen fermentation, reducing total VFA and ammonia N concentrations and showing a linear increase in the molar proportion of propionate and a quadratic effect on the molar proportions of acetate and butyrate.

The main propose of this experiment was conducted to find effects of clove methanol extract on methane production in ruminant with practical diets.

MATERIALS AND METHODS

Experimental location: This study was carried out as an *in vitro* assay using *Syzygium aromaticum* (clove) methanol extract on methane production in the laboratory of Islamic Azad University, Shabestar branch.

Plant materials and preparation of extracts: *Syzygium aromaticum* (clove) (Fig. 1) samples were collected, dried in a hot air oven at 55-60°C temperature, ground to pass through 1 mm sieve. One-hundred grams of clove with 1000 ml of methanol (98%) were shacked for 24 h and filtered through Whatman filter paper. Methanol soluble (methanol extract) fractions of clove were prepared as per the procedure adopted by Patra *et al.* (2006). All methanol extract samples were collected in individual sample container and kept under 4°C until used. Artificial saliva was produced using (Menke and Steingass, 1988) technique. The dried methanol extracts and residues were weighed and stored in air tight containers for further use and tested for their potency to reduce methanogenesis procedure.

Two experimental diets: Two experimental diets (diet 1: 100% forage and diet 2: 100% barley) were used to investigate effects of 3 levels of CME (0, 0.5, 1 mL) on methane production. Treatment A and D kept as a



Fig. 1: *Syzygium aromaticum* (fresh and dry)

control group without any CME additive whereas treatment was B: diet 1 with 0.5 mL CME; treatment C: diet 1 with 1 mL CME; treatment E: diet 2 with 0.5 mL CME and treatment F: diet 2 with 1 mL CME with three replicates for each treatment, respectively. Each experimental sample contains 20 ml artificial saliva and 10 ml rumen fluid (2: 1 v/v ratio), 200±10 mg experimental diet and CME levels.

In vitro gas production assay: The serum disposable bottles were gently shaken 30 min after the start of incubation and every hour for the first 10 h of incubation. Methane production was measured as the volume of gas in the calibrated serum disposable bottles and was recorded time of incubation 2, 4, 6, 8, 12, 24, 48, 72 and 96 h after incubation with GC (EX-TEC HS680). All samples were incubated in triplicate with three serum disposable bottles containing only rumen fluid-buffer mixture (blank).

Statistical analysis: Data on apparent gas production parameters were subjected to one-way analysis of variance using the analysis of variation model ANOVA using SAS (2003). Multiple comparison tests used Duncan's Multiple-Range Test (1980). All values were shown as standard error of difference between means (SEM).

RESULTS

Results of administration different levels of CME are showed in Table 1. All data was shown as percent. According to our results there was significant deference in methane production between the groups after 2, 4, 12, 16, 24, 48, 72 and 96 h of incubation.

Results show that CME could increase methane volume in all of experimental treatments. In the forage diet 0.5 and 1 ml CME significantly have deferent compared control group in the 2 h incubation time and from 1% in control group reached to 3 and 4% in the experimental treatment, respectively. And for the barley diet from 1% reached to 3 and 2% in the treatments, respectively. In the 4 h incubation approximately not different between treatment, but increased incubation time for the 12 h use of CME could decreased methane volume and in the 100% of forage diet from 8% reach to 2 and 4% and in the barley diet from 12% reached to 2 and 3%,

Table 1: Effects different levels of CME on methane production on the *in vitro* condition

Diets	Extract	Incubation times (h)							
		2	4	12	16	24	48	72	96
100% forage	(Control) 0	1 ^e	3 ^f	8 ^{hi}	9 ^{de}	7 ^c	14 ^b	9 ^{hi}	11 ^h
	0.5 ml	3 ^c	2 ^g	2 ^k	2 ^{ij}	1 ^g	1 ^m	1 ^j	2 ^{lm}
	1 ml	4 ^b	3 ^f	4 ^j	3 ^{hi}	2 ^{fg}	1 ^m	2 ⁱ	4 ^{lmk}
100% barley	(Control) 0	1 ^e	2 ^g	12 ^e	10 ^{dc}	12 ^a	10 ^{cd}	15 ^e	14 ^{fg}
	0.5 ml	3 ^d	2 ^g	2 ^k	1 ^j	1 ^g	1 ^m	1 ^j	1 ^m
	1 ml	2 ^c	3 ^f	3 ^{kl}	1 ^j	2 ^{fg}	3 ^{kl}	1 ^j	2 ^{lm}
p-value		<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
SEM		0.073	0.114	0.247	0.174	0.160	0.181	0.341	0.433

respectively. This condition shown in the other incubation time and 0.5 and 1 ml of CME could significantly decreased methane volume.

DISCUSSION

Global warming contributed by greenhouse gases which methane is one of them, by Johnson and Johnson (1995). Subsequently, this phenomenon due to e.g. melting poles ice and increase free water levels in the earth. Two-thirds of the anthropogenic gas population sources are Agriculture. Anaerobic digestion in ruminants is a major source of methane production compare to non-ruminant livestock. Ingested feeds degrade, digested and hydrolysis in the rumen to their ingredients e.g. proteins, amino acids, carbohydrates, lipids. Such anatomical characteristics with a small intestine flanked by two microbial compartments at both ends are much more efficient for the digestion of carbohydrates and for the degradation of plant cell walls. Furthermore, microbial protein synthesised in the forestomachs is then available for digestion in the small intestine where they supply more than 50% of the amino acids entering the blood stream. In the rumen, formation of methane is following through below reaction: $\text{CO}_2 + 4 \text{H}_2 \rightarrow \text{CH}_4 + 2\text{H}_2\text{O}$, Moss *et al.* (2000). Patra *et al.* (2005a,b) observed *in vitro*, using rumen fluid sampled from animals fed on roughage-based diets, that ruminal methanogens lose the ability to use H_2 at low pH, giving rise to free H_2 in the gas phase when the pH was less than 5.5. Thus on roughage diets a low pH leads to a decrease in methanogenesis independent from propionate formation. Sub-group of the anaerobes Archae domain producing methane in the rumen by Woese *et al.* (1990). Previous researchers demonstrated that using phenolic compound e.g. Tannins, Clove, Thymol as a feed additive could decrease methane production in ruminants by Patra *et al.* (2005a,b); Patra and Saxena (2010) and Hassanpour *et al.* (2011).

Eugenol (4-allyl-2-methoxyphenol; $\text{C}_{10}\text{H}_{12}\text{O}_2$) is a phenolic compound with wide-spectrum antimicrobial activity against gram-positive and gram-negative bacteria and it is one of the main active components in clove bud by Calsamiglia *et al.* (2007). In the current study we use Clove methanol extract to determining methane production as an *in vitro* assay. Our results

indicated that methane production decreased by additive CME in both of experimental diets at 12, 16, 24, 48, 72 and 96 h of incubation. We believe, Eugenol has antimicrobial effect on methanogenesis bacterial. In recent year's researchers found that ruminal methane production reduce by Plant Secondary Metabolites as safe ruminal fermentation modulators by Sirohi *et al.* (2009). Similar findings were reported by Patra *et al.* (2005a,b) which Clove reduces methane production in rumen. In another study, Busquet *et al.* (2005) observed low doses of clove bud oil (2.2 mg/L) due to lower proportions of acetate compare higher molar proportion of propionate. Generally, in starch rich diets which favour propionate production will decrease the methane in the rumen. As discussed before, the effect of such diets on ruminal pH can also explain the observed effect on methane emission. Conversely, a roughage-based diet will increase the ratio some other feed characteristics can affect methane production by Moss *et al.* (2000). Also Patra *et al.* (2006) reported that seed pulp methanol extract of Terminalia chebula and methanol, ethanol and water extracts of Allium sativum bulbs were significantly reduced methane production in rumen liquor of buffaloes. Our results indicated that CME could decrease Methane production in rumen. Also, by passing time we saw significantly reduction in methane production. We believe CME decrease methane production with 2 ways. One of ways is reducing methanogenesis bacteria population by antimicrobial effects of Eugenol. The other way is reducing methane production by shift H_2 protons to propionate production as acetate and butyrate production. So, in this situation CO_2 couldn't complex with H_2 and due to decrease methane production. We suggested CME is suitable substance as a feed additive in ruminant nutrition. Also, there are more researches needs to found any anti-nutritional effects of Clove on ruminants.

Conclusion: Active principles of herbs having capacity to reduce methane production differ in their property of solubility. The active principles involved in reducing methane production are not water soluble. According to the results of this study, we believe that the CME have the potential affect on ruminal fermentation efficiency and CME could be a promising substance in methane mitigating agent.

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