In vitro Evaluation of Sheep Rumen Fermentation Pattern After Adding Different Levels of Eugenol – Fumaric acid Combinations

T A M Baraka[1*] and M A Abdl-Rahman[2]

1. Department of Medicine and Infectious Diseases,
2. Dept. of Physiology, Faculty of Veterinary Medicine, Cairo University, Giza, 12211, Egypt.
*Corresponding author Email: drtaherbaraka@yahoo.com
Received: 13-08-2011, Accepted: 24-09-2011, Published Online: 05-12-2011
doi: 10.5455/vetworld.2012.110-117

Abstract

In vitro gas production technique was used to evaluate the effect of three different levels of eugenol + fumaric acid combinations on rumen fermentation. Rumen contents were collected from five rams immediately after slaughtering and used for preparation of inoculums of mixed rumen microbes that were used in generation of five treatment systems, negative control with no additives (T1), fumaric acid 0.5 mg L^{-1} (T2) and fumaric acid 0.5 mg L^{-1} in combination with three different doses of eugenol, 100, 200 and 400 mg L^{-1} (T3, T4 and T5 respectively). Incubations were conducted in triplicates with gas production, pH, ammonia nitrogen (NH_3-N), total and fractional volatile fatty acids (VFAs) concentrations, cellulase activity, amount of substrate degraded, microbial yield (Y_{ATP}), fermentation efficiency (FE) and VFAs utilization index (NGGR) were determined after 24 hours of incubation. The results revealed that, different levels of eugenol + fumaric acid combinations were associated with decreased pH value, NH_3-N concentrations and methane production and increased valeric and isovaleric acids molar proportions. T3 and T4 were associated with increased propionates at the expence of acetates (low A/P), decreased methane production and increased FE, microbial yield (Y_{ATP}) and VFAs utilization. In contrast, T5 showed decreased total VFAs concentrations, cellulase activity, the amount of substrate degraded, microbial mass generated and VFAs utilization. In conclusion, the authors recommend using 200 mg L^{-1} eugenol + fumaric acid combination as an alternative for antibiotic feed additives to optimize rumen fermentation pattern. Further investigations are required to apply this work *in vivo* experiments.
Keywords: Eugenol, Fermentation efficiency, Fumaric acid, Rumen microbes.

To cite this article : Baraka TAM and Abdl-Rahman MA (2012): *In vitro* Evaluation of Sheep Rumen Fermentation Pattern After Adding Different Levels of Eugenol – Fumaric acid Combinations, *Vet. World* 5 (2): 110-117, doi: 10.5455/vetworld.2012.110-117

Introduction

Manipulation of rumen microbial fermentation to decrease methane and ammonia nitrogen production using antibiotic feed additives has proved to be a useful strategy to improve production efficiency in ruminants (McGuffey *et al.*, 2001). However, the use of antibiotics as feed additives in ruminants is banned in the European Union because of the risk of residues (Russell and Houlihan, 2003) and emergence of multi-drug resistant bacteria (Gustafson and Bowen, 1997) that may threat human health. This risk fueled the search for non-antibiotic alternatives, which might have similar effects on animal performance.

Among these alternatives, essential oils and fumaric acid are most hopeful and have received much attention. Essential oils are secondary plant metabolites that generally recognized as safe for human consumption (FDA, 2004) and able to modify rumen microbial fermentation through a wide spectrum of antimicrobial activity (Greathead, 2003) especially on Gram-positive bacteria (Burt, 2004). Phenolic components such as eugenol (one of the components of clove) are responsible for the antibacterial properties of many essential oils (Dorman and Deans, 2000), and appear to act as membrane permeabilizers (Helander *et al*., 1998).

The impact of a blend of feed additives that are currently registered for feeding to ruminants on rumen fermentation pattern can be applied using *In vitro* gas production technique which is proved to be a potentially appropriate and well correlated with rumen fermentation pattern, microbial protein synthesis, *in vivo* digestibility and animal performance (Kamalak *et al*., 2005).

Several studies showed that eugenol could manipulate rumen fermentation presumably VFAs profile, ammonia N concentrations and gas production (Busquet *et al*, 2006; Castillejos *et al*., 2006; Benchaar *et al*., 2007).

Fumaric acid is an organic dicarboxylic acid (C4H4O4) authorised for all animals in all feeding stuffs without maximum level. Fumaric acid is a metabolic precursor of propionate (Newbold and Rode 2006), and provide an alternative hydrogen sink within the rumen (Itabashi, 2002). However, its maximum potential to divert H_2 away from CH_4 is limited presumably because methanogen utilize H_2 more rapidly than fumarate-utilizing bacteria (Asanuma *et al.*, 1999).

The objective of this in vitro study was to investigate effects of adding fumaric acid together with three different doses of eugenol using *In vitro* gas production technique to achieve auxiliary optimistic effects on rumen fermentation pattern.

Materials and Methods

This investigation was conducted in Department of Physiology and Rumenology laboratory in Department of Medicine and Infectious Diseases, Faculty of Veterinary Medicine, Cairo University, Egypt. Rumen contents used in preparation of the treatment systems were collected from five recently slaughtered rams and strained through four layers of cheesecloth into a separating flask previously gassed with oxygen-free CO_2 and brought immediately to the laboratory. Strained rumen liquor was mixed with the buffer solution of Goering and Van Soest (1970) in the proportion 1:2 (v/v), flushed with oxygen-free CO_2 and used as inoculums of mixed rumen microorganisms. Part of the buffered rumen fluid sample (blank) was not used as inoculum, immediately mixed with 0.3 mL H_2SO_4 10N and used for determination of total VFAs concentration before incubation.

Preparation of treatment systems and *in vitro* fermentation: The method used for *in vitro* fermentation was based on the *in vitro gas production* technique described by Menke and Steingass (1988). Two- hundred milligrams of feed sample (Composition and chemical analysis is shown in Table 1), previously ground with a pestle and mortar, were carefully dropped into 100 ml calibrated plastic syringes with pistons lubricated with vaseline. Thereafter, 30 ml of buffered rumen fluid were dispensed into the syringes and the following treatment systems were then prepared in triplicates under continuous flushing with CO_2, negative control with no additives (T1), fumaric acid 0.5 mg L^{-1} (T2) and fumaric acid 0.5 mg L^{-1} in combination with three different doses of eugenol, 100, 200 and 400 mg L^{-1} (T3, T4 and T5 respectively).

Table-1. Composition and chemical analysis of the used basal diet

Ingredients	% as fed
Barely grain	38.06
Berseem hay	39.70
Wheat bran	21.14
Salt	0.50
Vitamin and mineral premix	0.30
DL-Methionine	0.30
Chemical analysis	**% of dry matter**
Crude fibers	31.00
Crude proteins	13.80
Ether extract	2.80
Nitrogen free extract	34.50
total ash	8.60
Digestible energy (MJ/Kg diet)	8.80

Eugenol doses were previously prepared as stock solutions by dissolving eugenol in 99.5% ethanol and a total of 0.2 ml of each dose was added to the respective syringes. Equivalent amounts of ethanol (0.2 ml) were added to the syringes assigned for control and uncoupled fumaric acid treatments. After gentle shaking, syringes were tapped and pushed upward by the piston in order to completely eliminate air in the inoculums; the tips were then tightened by a metal clip so as to prevent escape of gases.

Table-2. Effect of treatment systems on fermentation pattern by mixed rumen micro-organisms after 24 hours in vitro incubation (measured parameters)

Parameters	T1	T2	T3	T4	T5
pH value	6.62 [a] ± 0.00	6.64 [a] ± 0.00	6.36 [b] ± 0.03	6.36 [b] ± 0.03	6.38 [b] ± 0.04
Total VFAs (µmol)	758.0 [a] ± 15.9	760.6 [a] ± 14.1	774.6 [a] ± 11.6	775.6 [a] ± 13.6	659.3 [b] ± 26.0
Acetic acid (mol%)	51.00 [a] ± 0.22	49.26 [a] ± 0.05	46.12 [b] ± 0.46	43.13 [c] ± 0.25	45.00 [b] ± 0.29
Propionic acid (mol%)	26.1 [a] ± 0.21	27.7 [b] ± 0.31	28.9 [c] ± 0.36	30.8 [d] ± 0.35	24.1 [e] ± 0.11
Butyric acid (mol%)	17.0 [a] ± 0.26	17.7 [a] ± 0.32	17.6 [a] ± 0.35	17.8 [a] ± 0.21	22.8 [b] ± 0.35
A / P ratio	1.84 [a] ± 0.12	1.78 [a] ± 0.02	1.59 [b] ± 0.03	1.40 [c] ± 0.01	1.86 [a] ± 0.01
Valeric (mol%)	3.03 [a] ± 0.3	2.49 [a] ± 0.2	3.46 [ab] ± 0.2	3.94 [ab] ± 0.3	4.10 [b] ± 0.4
Isovaleric (mol%)	2.2 [a] ± 0.7	1.6 [a] ± 0.3	2.4 [b] ± 0.1	2.7 [b] ± 0.2	2.6 [ab] ± 0.1
Isobutyric (mol%)	1.0 ± 0.28	1.1 ± 0.14	1.4 ± 0.1	1.5 ± 0.05	1.4 ± 0.23
Ammonia N. (mg dL–1)	11.73 [a] ± 0.33	11.58 [a] ± 0.33	9.11 [b] ± 0.22	9.06 [b] ± 0.39	8.76 [b] ± 0.29
Gas volume (ml)	23.7 ± 0.53	23.8 ± 0.44	23.0 ± 0.58	21.8 ± 0.60	22.0 ± 0.76
Cellulase activity*	5.67 [a] ± 0.15	5.53 [a] ± 0.10	5.63 [a] ± 0.15	5.57 [a] ± 0.09	3.74 [b] ± 0.42
Amount of substrate**	73.5 [a] ± 0.5	74.8 [ab] ± 0.3	75.9 [b] ± 0.5	76.8 [b] ± 0.4	66.3 [c] ± 1.1

Data presented as means ± SE, N =5 (prepared in triplicates), Values having different letters in the same raw are significantly different at P> 0.05, *Cellulase activity (mmol glucose eq/ min) ** Amount of substrate truly degraded (mg)

Incubation was carried out at 39°C and the volume of gas produced was recorded after 24 hours (The degradation time in the rumen needs 48 hours). After termination of incubation, the fluid samples were drawn into plastic bottles and pH values were immediately measured using a digital pH meter. Supernatant from each fluid sample was separated by centrifugation at 7,000 x *g* for 10 minutes. For determination of total VFAs concentrations and individual VFAs proportions, one mL of 25% meta-phosphoric acid was added to 5 ml of supernatant and stored at -20°C until analyzed. For NH_3–N determination, two milliliters of supernatant was acidified with 2 ml of 0.2 N HCl and were analyzed by spectrophotometry for NH_3–N according to Chaney and Marbach (1962).

Total VFAs concentrations were measured by steam distillation according to Eadie *et al.* (1967). The VFAs concentrations were analyzed using High Performance Liquid Chromatography (HPLC; Model Water 600; UV detector, Millipore Crop.) according to the method of Mathew *et al.* (1997) and following fermentation parameters were calculated:

1- Amounts of VFAs produced were obtained by subtracting the amount present initially in the incubation medium (blank) from those determined at the end of the incubation period.
2- Acetic / propionic acid ratio (A/P).
3- Fermentative CH_4 production in the buffered rumen fluid was estimated according to the equations of Wolin (1960), which has been validated recently by Blummel *et al.* (1993).
4- Percent of methane output per total VFAs production.
5- Fermentation efficiency (FE) was calculated on the basis of the equation worked out by Orskov (1975) and modified by Baran and Zitnan (2002).
6- VFAs utilization index was expressed by non-glucogenic VFAs/glucogenic VFAs ratio (NGGR) and estimated according to Orskov (1975).

Measurement of cellulase activity: From each crude enzyme solution of the supernatant 0.5 ml was mixed with 0.5 ml of 1% carboxymethyl cellulose solution in 0.05 M sodium citrate buffer. The reaction proceeded for one hour at 55°C without shaking, and then stopped by boiling for 5 min. Boiled samples were centrifuged at 7,000 x *g* for 5 min, and reducing sugars produced in the supernatant was measured colorimetrically according to Miller *et al.* (1960). One unit of enzyme activity was defined as the amount of enzyme that produced 1 mmol of glucose equivalent of reducing sugar per minute.

Measurement of in vitro true degradability and microbial mass generated: *In vitro* true degradabilities were determined according to the procedures of Blummel *et al.* (1997). Microbial mass generated by termination of incubation was then estimated according to Grings *et al.* (2005).

Table-3. Effect of treatment systems on fermentation pattern by mixed rumen micro-organisms after 24 hours in vitro incubation (calculated parameters)

Parameters	T1	T2	T3	T4	T5
CH4 (µmol)	208.7 [a] ± 5.9	202.2 [a] ± 5.2	187.6 [b] ± 4.4	172.3 [b] ± 2.6	183.9 [b] ± 9.3
CH4 /total VFAs (%)	27.5 [a] ± 0.21	26.5 [b] ± 0.26	24.6 [c] ± 0.49	22.8 [d] ± 0.07	27.8 [a] ± 0.30
FE (%)	77.0 [a] ± 0.0	77.8 [b] ± 0.1	78.6 [c] ± 0.2	79.6 [d] ± 0.1	77.4 [e] ± 0.0
NGGR	3.03 [a] ± 0.02	2.89 [a] ± 0.05	2.62 [b] ± 0.07	2.38 [c] ± 0.01	3.63 [d] ± 0.06
Microbial mass (mg)	21.25 [a] ± 0.68	22.43 [a] ± 0.2	25.33 [b] ± 1.16	28.83 [c] ± 0.95	17.63 [d] ± 0.47
Microbial yield (YATP)	10.86 [a] ± 0.65	11.26 [a] ± 0.22	13.01 [b] ± 1.07	14.98 [b] ± 0.66	10.59 [a] ± 0.85

Data presented as means ± SE, N = 5 (prepared in triplicates)
Values having different letters in the same raw are significantly different at P> 0.05

Microbial yields (Y_{ATP}) were calculated as the mg microbial mass produced per mmole ATP generated in fermentation of carbohydrates to VFAs. Moles of ATP generated per mole of short chain VFAs and methane are 2 for acetate, 3 for propionate, 3 for butyrate, and 1 for methane (Isaacson *et al.*, 1975).

Obtained data were statistically analyzed using the SPSS Statistical Computer Software, Copyright (c) SPSS Inc., 2007 version 16.0 based on one-way ANOVA, with *post hoc* Duncan multiple comparison test. Differences at $p < 0.05$ were considered significant.

Results

Data presented in tables 2 and 3 showed that, the effects of eugenol + fumaric acid combinations on rumen fermentation pattern were different depending on the dose. Table 2 revealed that, the pH value of the fermentation fluid was decreased by all levels of eugenol + fumaric acid combinations relative to control. Adding fumaric acid singly or in combinations with eugenol at 100 and 200 mg L^{-1} levels did not alter total VFAs concentrations but when was added with eugenol at 400 mg L^{-1} level resulted in reduced VFAs concentrations. The means of VFAs molar proportions identify that, T2, T3 and T4 were associated with increased propionate at the expense of acetate without detectable alteration in butyrate molar proportions. In contrast, T4 was associated with increased butyrates at the expense of both acetates and propionates. Furthermore, the molar proportions of the major branched chain VFAs (valeric–isovaleric - isobutyric) were not affected by addition of fumaric acid singly but combinations with different levels of eugenol achieved an increase in valeric and isovaleric acids molar proportions. It is worthy of note that the decremental and incremental effects in T4 surpassed those induced by other eugenol levels. Concerning NH_3-N concentrations, combinations of fumaric acid with different levels of eugenol induced a decremental effect with reference to control, while, addition of fumaric acid alone did not alter NH_3-N concentrations.

Extracellular cellulase activity within the fermentation fluid and the amount of substrate degraded did not alter by different treatment systems relative to control except for the recorded reduction in T5.

Methane production was greatly reduced by the different levels of fumaric acid - eugenol combinations (table-3) and this was most obvious in T4. Means of the calculated fermentation efficiencies reveal the following order: T4 > T3 > T2 > T1, while T5 did not alter fermentation efficiency with reference to control. In contrast, NGGR was decreased in T3 and T4, increased in T5 and did not differ by addition of fumaric acid singly in T2. Furthermore, microbial mass generated during fermentation was increased in T3 and T4, decreased in T5 while, it also was not affected by addition of fumaric acid singly (T2). Relative to control, microbial yield / mmole ATP generated during fermentation (Y_{ATP}) was increased in T3 and T4 while, it was not affected by addition of fumaric acid singly or in combination with eugenol at 400 mg L^{-1} level.

Discussion

Results reveal that the effects of eugenol + fumaric acid combinations on rumen microbial activity varied greatly with regard to dosage of

eugenol added. Presence of a hydroxyl group in the phenolic structure of eugenol is responsible for its high antimicrobial activity (Dorman and Dean, 2000; Burt, 2004) and hence its dose-dependent properties. T3 and T4 achieved additional improvements in VFAs profile towards increased propionates at the expense of acetates, while at T5 combination, the results were frustrating as both acetic and propionic acids were reduced relative to control. It is predictable that, each mole of fumaric acid when converts into propionic acid would stoichiometrically decrease CH_4 production by 5.6 liters (Newbold *et al*., 2005); the inability of fumarate-reducers to compete for H_2 with methanogens limits the efficacy of fumaric acid to play this role (Asanuma *et al*., 1999). The additional increase in propionic acid proportions achieved in T3 and T4 corresponded well to the additional decrease in CH_4 production by these treatments relative to uncoupled addition of fumaric acid. This result confirms the issue that hydrogen diverted away from methane is used in reduction of fumarates into propionates with little production of acetates and butyrates (Bayaru *et al*., 2001).

The pattern of VFAs observed in T5 mix could stem from inhibition of gram-negative, propionate-producing bacteria and activation of gram-positive, butyrate-producing bacteria. Burt (2004) suggested that gram-positive bacteria appear to be more susceptible to the antibacterial properties of plant extracts than gram-negative bacteria. Similar VFA patterns were observed by Benchaar *et al*. (2007) when used eugenol at the concentration of 800 mg L^{-1}, while at 500 mg L^{-1} eugenol, Castillejos *et al*. (2006) observed no effect on the molar proportions of acetate and butyrate, but traced a decrease in propionate molar proportion. Busquet *et al*. (2006) observed that when used at the concentration of 300 mg L^{-1}, eugenol did not change molar proportions of acetate and propionate, but increased the proportion of butyrate.

Therefore, this VFA pattern suggests that, in T5 combination, gram-negative bacteria were more sensitive to the antibacterial activity of eugenol than gram-positive bacteria. Furthermore, the recorded reduction in total VFA concentration at this level warns that high doses of eugenol are likely to be detrimental for rumen microbial fermentation if the same effects were expressed *in vivo*. Increased concentrations of both valeric and isovaleric acids at all levels of eugenol + fumaric acid combinations indicates enhanced microbial deaminative activity as deamination of branched chain amino acids represents the major source of BCVFAs (Hino and Russell, 1985). This result is analogous to that obtained by Busquet *et al*. (2006) when added eugenol at the concentration of 300 mg L^{-1} but incosistent to that observed by Castillejos *et al*. (2006) when used eugenol at 500 mg L^{-1} concentration. More puzzling is the tendency to lower NH_3-N concentrations associated with all levels of fumaric acid + eugenol combinations despite the suggested increase in deaminative activity. However, in T3 and T4 combinations, these low NH3-N concentrations could be attributed to greater ammonia utilization by rumen microbes as both microbial mass and microbial yield (Y_{ATP}) were increased in accordance to the decremental effect of each of the previous treatments. Reduction of methane production by all levels of fumaric acid - eugenol combinations was efficient especially in T4 combination and this probably saved more energy to meet the synthetic needs of rumen microbes. Huber and Herrara (1994) suggested that a synchronous supply of energy and NH_3-N is required for ammonia utilization in microbial protein synthesis.

The substantial microbial cell yield (Y_{ATP}) achieved in T3 and T4 combinations could also be related to the additional increase in propionate at the expense of acetates associated with these combinations. Leng (1993) suggested the possession of a system for ATP regeneration by electron transport phosphorylation coupled with propionate producing bacteria but absent in acetate producing bacteria, which implies that these bacteria acquire energy from hydrogen ions which otherwise are used to produce methane as suggested by Asanuma *et al* (1999). The recorded pH values here were within the normal range required for optimum microbial activities

(Russell and Welson, 1996). VFAs and ammonia concentrations of the fermentation fluid are the principal determinants of pH values, as they are the main sources of H^+ and OH^- and hence, reduction of pH associated with all levels of fumaric acid - eugenol combinations could be attributed to the recorded reduction in NH_3-N concentrations.

Cellulase activity and the amount of substrate degraded did not alter in T3 and T4 combinations which informs on efficient H_2 disposal without negative drawbacks on cellulolytic bacterial activity. Recorded reduction associated with T5 combination points to harmful effects on cellulolytic bacterial activity. The fermentation efficiency calculated for T4 combination amounted to 79.6% and outperformed the T3 combination by 1.27% and the uncoupled fumaric acid addition by 2.3% whereas; T5 combination exerted a damping effect on fermentation efficiency. Calculation of fermentation efficiency is based on conversion of hexose energy to VFAs energy on the basis of equations worked out by Orskov (1975) and modified by Baran and Zitnan (2002). It is clear that, increased fermentation efficiency achieved by T3 and T4 combinations is actually the end result of their detrimental effect on methane production, their ability to increase propionate the expense of acetates and butyrates and accordingly their ability to enhance ammonia utilization in microbial protein synthesis.

In contrast to fermentation efficiency, VFAs utilization index (NGGR) being optimum at values amounts to 3.5 and higher values indicate the worse use of VFA (Czerkawski, 1986). The lowest value of NGGR, which indicates the best utilization of VFAs, was achieved by T3 and T4 combinations (2.62 and 2.38 respectively) that imply an improvement in VFAs utilization by about 9.34% and 17.6% respectively relative to uncoupled fumaric acid addition (T2). As well, the T5 combination gave the lowest VFAs utilization. The detrimental effect of T5 combination on both fermentation efficiency and VFAs utilization corresponds well to its previously mentioned subordinate impact on total and fractional VFAs concentrations, cellulase activity, amount of substrate degraded and microbial yield (Y_{ATP}).

Conclusion

Form the three different levels of eugenol - fumaric acid combinations tested in this study, only 100 and 200 mg L^{-1} eugenol + fumaric acid combinations improved VFAs profile, VFAs utilization, microbial cell yield and rumen fermentation efficiency. High doses of eugenol (400 mg L^{-1}) could be detrimental on rumen microbial fermentation. It is advisable to use the 200 mg L^{-1} eugenol + fumaric acid combination as an alternative for antibiotic feed additives to optimize rumen fermentation pattern. Further investigations are required to apply this work *in vivo* experiment.

Acknowledgements

Authors express thanks to the support given by research project: Banking of Rumen Ciliates for Improvement of Ruminant Farm Animals Production; financially supported by Cairo University Research Sector and P. I. Dr. Baraka, T. A. and his laboratory team for analysis of samples collected along the experiment.

Conflict of interest

Authors declare that they have no conflict of interest.

References

1. Asanuma A., Iwamoto M., Hino T. (1999). Effect of the addition of fumarate on methane production by ruminal micro-organisms in vitro. *Journal of Dairy Science.* 82: 780.
2. Baran M., Zitnan R. (2002). Effect of monensin sodium on fermentation efficiency in sheep rumen. *Arch. Tierzucht.* 45:181.
3. Bayaru E., Kanda S., Kamada T., Itabashi H., Andoh S., Nishida T., Ishida M., Itoh T., Nagara K., Isobe Y. (2001). Effect of fumaric acid on methane production, rumen fermentation, digestibility of cattle fed roughage alone. *Anim. Sci.* 72: 139.
4. Benchaar C., Chaves A. V., Fraser G. R., Wang Y., Beauchemin K. A., McAllister T. A. (2007). Effects of essential oils and their components on in vitro rumen microbial

fermentation. *Can. J. Anim. Sci.* 87:413.
5. Blummel M., Steingass H., Becker K., Soller H. (1993). Production of SCFA, CO2, CH4 and microbial cells in vitro. *Proceedings of the Society of Nutrition Physiology.* 1: 9.
6. Blummel M., Makkar, H. S., Becker K. (1997). In vitro gas production: a technique revisited. *J. Anim. Physiol. Anim. Nutr.* 77:24.
7. Burt S. (2004). Essential oils: their antibacterial properties and potential applications in foods – a review. *Int. J. Food Microbiol.* 94:223.
8. Busquet M., Calsamiglia S., Ferret, A., Kamel, C. (2006). Plant extracts affect in vitro rumen microbial fermentation. *J. Dairy Sci.* 89:761.
9. Castillejos L., Calsamiglia S., Ferret A. (2006). Effect of essential oil active compounds on rumen microbial fermentation and nutrient flow in in vitro systems. *J. Dairy Sci.* 89:2649.
10. Chaney A. L., Marbach E. P. (1962). Modified reagents for determination of urea and ammonia. *Clinical Chemistry.* 8:130.
11. Czerkawski J. W. (1986). An introduction to rumen studies, *Pergamon Press, Oxford*, 236 pp.
12. Dorman J. J. D., Deans, S. G. (2000). Antimicrobial agents from plants: Antibacterial activity of plant volatile oils. *J. Appl. Microbiol.* 88:308.
13. Eadie J. M., Hobson P. N, Mann, S. O. (1967). A note on some comparisons between the rumen content of barley fed steers and that of young calves also fed on high concentrate rations. *Animal production* 9:247.
14. FDA. (2004). Food and Drug Administration of the US, 21 CFR 184. *http://www.cfsan.fda.gov/FCF184.html Accessed Sep. 20, 2004*.
15. Goering H. K., Van Soest P. J. (1970). Forage Fiber Analysis. *Agricultural Handbook* no. 379. Washington, DC: Agricultural Research Service, US Department of Agriculture.
16. Greathead H. (2003). Plants and plant extracts for improving animal productivity. *Proc. Nutr. Soc.* 62:79.
17. Grings E. E., Blummel M., Sudekum K.H. (2005). Methodological considerations in using gas production techniques for estimating ruminal microbial efficiencies for silage based diets. *Anim Feed Sci. Technol.* 123:527.
18. Gustafson R. H., Bowen R.E. (1997). Antibiotic use in animal agriculture. *J. Appl. Microbiol.* 83:531.
19. Helander I. M., Alakomi H., Latva-Kala K., Mattila-Sandholm T., Pol I., Smid E. J., Gorris L. G. M., Wright A. (1998). Characterisation of the action of selected essential oil components on gram-negative bacteria. *J. Agric. Food Chem.* 46:3590.
20. Hino T., Russell J. B. (1985). Effect of reducing–equivalent disposal and NADH/NAD on deamination of amino acids by intact rumen microorganisms and their cell extract. *Applied Environmental Microbiology*. 50:1368.
21. Huber J. T., Herrara R. (1994). Synchrony of protein and energy supply to enhance fermentation. In: Asplund, J. M., (Ed), *Principles of protein nutrition of ruminants*. Columbia, USA, pp. 113-136.
22. Isaacson H. R., Hinds, F.C.; Bryant, M.P.; Owens, F.N. (1975): Efficiency of energy utilization by mixed rumen bacteria in continuous culture. *J Dairy Sci.* 58. 1645-1659.
23. Itabashi H. (2002). Reducing ruminal methane production by chemical and biological manipulation. In: Takahashi J., Young B. A. (Eds), Greenhouse Gases and Animal Agriculture. Elsevier, Amsterdam, *The Netherlands.* pp. 139.
24. Kamalak O., Canbolat Y., Gurbuz O., Ozay (2005). Comparison of in vitro gas production technique with in situ nylon bag technique to estimate dry matter degradation. *Czech J. Anim. Sci.* 50:60.
25. Leng R. A. (1993). Quantitative ruminant nutrition - A green science. *Aust. J Agric. Res.* 44**:**363.
26. Mathew S., Sagathevan S., Thomas J,

Mathen G. (1997). An HPLC method for estimation of volatile fatty acids in ruminal fluid. *Indian Journal of Animal Sciences.* 67:805.
27. McGuffey R. K., Richardson L. F., Wilkinson J. I. D. (2001). Ionophore for dairy cattle: Current status and future outlook. *J. Dairy Sci.* 84:194.
28. Menke K. H., Steingass H. (1988). Estimation of the Energetic Fed Value from Chemical Analysis and In Vitro Gas Production Using Rumen Fluid. *Anim. Res. Dev.* 28:7.
29. Miller J. L., Blum R., Glennon W. E., Burton A. L. (1960). Measurement of carboxy-methyl cellulase activity. *Analytical Biochemistry* 1:127.
30. Newbold C. J., Lopez S., Nelson N., Ouda J. O., Wallace R. J., Moss A. R. (2005). Propionate precursors and other metabolic intermediates as possible alternative electron acceptors to methanogenesis in ruminal fermentation *in vitro*. *British Journal of Nutrition*. 94:27.
31. Newbold C. J., Rode L. M. (2006). Dietary additives to control methanogenesis in the rumen. *International Congress Series.* 1293:138.
32. Orskov E. R. (1975). Manipulation of rumen fermentation for maximum food utilization. *World Reveiw Nutrition Diet.* 22:153.
33. Russell J. B., Houlihan A. J. (2003). Ionophore resistance of ruminal bacteria and its potential impact on human health. *FEMS Microbiol. Rev.* 27:65.
34. Russell J. B., Welson R. J. (1996). Why are ruminal cellulolytic bacteria unable to digest cellulose at low pH? *Journal of Dairy Science*. 79:1503.
35. Snedecor G. W., Cochran, W. G. (1980). *Statistical methods*. 7th edition, Oxford and J.B.H. puplishing co.
36. Wolin M. J. (1960). A Theoretical rumen fermentation balance. *Journal of Dairy Science.* 43:1452.

* * * * * * * *