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Ability of Mentha puelgium and Thymus algeriensis essential oils to reduce methanogenosis in cheep: in vitro study

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ABSTRACT

The methane (CH_4) is a gas produced in large quantities in the rumen by degradation of food ration performed by methanogenic archaea and protozoa. Efforts have been made to reduce the methane emissions by ruminants, which is advantageous for nutritional and environmental perspective. The aim of this study was to investigate the influence of essential oils (EO) extracted from *Thymus algeriensis* and *Mentha pulegium* on the methane production and the rumen digestibility.

The extraction of EO from the plants was made by steam distillation. To study the effect of these EO on the rumen methanogenesis and the digestibility of the dray and organic matter, the method of Menke and Stingass (1988) was adopted using three doses (16.6, 33.3 and 66.6µl).

The results of this study indicate that EO decreased the production of methane after 72 hours of fermentation for the different doses. This decrease the number of protozoa on which the methanogenic archaea was grafted. The results of the digestibility showed that EO do not affect the digestibility of the dray and organic matter of the oaten vetch hay. These results are very important because they can justify partially the use of *Mentha pulegium* and *Thymus algeriensis* in the ruminant alimentation.

KEYWORDS

Methane;
Essential oils;
In vitro fermentation;
Digestibility.

INTRODUCTION

Ruminants are polygastric mammals which are distinguished by three additional digestive organs at the anterior end of the tract, namely the rumen, reticulum and omasum[22]. Like other ruminants, sheep are devoid of digestive enzymes to digest fibrous plant material. The digestion of these elements starts by fermentation which take place in the rumen[22].

Microbial fermentation in the rumen lead to the formation of volatile fatty acids and several gases mainly carbon dioxide (CO_2) and methane (CH_4). Methane is the principal route of elimination of hydrogen produced in the rumen during microbial digestion of food[32]. This gas represents an energy loss of 2–15% of the gross energy intake and it is a gas with very powerful greenhouse effect[9, 10]. Its contribution to the global warming is estimated at approximately 3%[19].

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Different strategies are deployed to mitigate methane emissions by ruminants. They are essentially interested to manipulate ruminal fermentation patterns and/or change of the equilibrium of the microbial population. Thus, the use of synthetic molecules regulators of microbial activity (antibiotics, halogenated molecules, …etc.) are used extensively for several years[9,; 22; 29]. However, the new European legislation and the recommendations of consumer protection organizations greatly limited the use of these methods. Recent legislation (1831/ 2003; EC, 2003) has been introduced within the European Union to prohibit the use of growth-promoting antibiotics in animal feeds which has led to an increased interest of natural substitutes like plants and their extracts[22].

Some of these new additives are the essential oils (EO). These are volatile and aromatic compounds with an oily appearance, which are obtained from plants[11]. Some work *in vitro* (artificial rumen) showed that they are effective in decreasing the amount of methane produced by bacteria in the rumen[6, 7, 16, 20, 24, 29, 40, 44].

The aim of this study is to explore the anti-methanogenic activity of essential oils extracted from *Thymus algeriensis* and *Mentha pulegium* in sheep, as well as an assessment of the ration digestibility in absence and in presence of these essential oils.

MATERIAL AND METHODS

Plant material

Two plants have been selected for extraction of their essential oils: *Mentha pulegium* and *Thymus algeriensis*. The identification of these two plants was made on the basis of Quezel and Santa (1963) key. The aerial parts of *M. pulegium* and *T. algeriensis* were collected during the month of may in the areas of BEKKARIA and SERDIES respectively (in Tebessa/Algeria).

Animal material

Ruminal fluid was obtained from three Sheep chosen randomly before being sacrificed in the slaughterhouse of Tebessa. Their diet is free and not defined. The rumen fluid was transferred to the laboratory into two prewarmed thermos flasks, preheated to 39 °C, squeezed through four layers of cheesecloth and purged with CO_2.

Extraction of essential oils

Extraction of essential oils was made by steam distillation. It was conducted for 3 to 4 hours from a mixture of 100 g of plant material and 1.5 L of distilled water into. Just after the extraction, the EO have stored in well closed glass bottles at 4°C and in darkness.

In vitro gas production study

The estimates of gas production were obtained by the method of Menke and Steingass (1988) by incubation in rumen fluid. All incubations were completed in 60 ml calibrated syringes containing 200 mg DM of oaten vetch hay and 30 ml of buffered rumen fluid (10mL rumen fluid and 20mL buffer solution). The piston was fitted precisely and lubricated using a small amount of Vaseline. The needle of the syringe was connected with a silicon rubber tube and closed using a plastic clip. The solution containing buffer solution and the macro and microminerals was prepared the day previous to incubation and stored at 39°C.

EO were added at doses of 0, 16.6, 33.3 and 6.66 µL in 30 mL of buffered rumen fluid under continuous flushing with CO_2. Triplicate syringes were used for each sampling. The syringes were incubated in a water bath (39 °C) for 72 h. At 2, 4, 8, 24, 48, and 72 h of incubation, the gas production (GP) was recorded. The methane concentration was determined after 24 and 72 h of incubation. Results were corrected for a blank incubation (i.e., buffered rumen fluid without sample) and oaten vetch hay standard at 24 h of incubation. After 24 h, the incubation the inoculants were determined and protozoa counts.

Rumen protozoa counts

In this study the protozoa were counted according to the method developed by Ogimoto and Imai (1981). After 24 hours of incubation, 100µl of buffered rumen fluid in each syringe was mixed with 100µl of methylgreen-formaldehyde-saline solution. This solution allows the fixation of cells by formaldehyde and colouring their nuclei by methyl green. The mixture is homogenized and kept in the dark for 30

minutes before counting. 1µl of this mixture was pipetted in Malassez cell. The protozoa were then counted using microscopy (x40). Each sample was counted twice, and if the average of the duplicates differed by more than 10%, the counting were repeated.

Study of the digestibility

Determination of the dry matter digestibility

After 72 hours of fermentation, the content of each syringe is centrifuged at 12000 rpm for 20 min and 4°C. The centrifugation nerve is dried at 60°C for 48 hours. The dry matter (DM) digestibility coefficient is calculated as follows:

D (%) = [IDM-(RDM-RDM) / IDM] * 100

Where: D: Digestibility

IDM: initial dry matter.

RDM: residual dry matter after incubation.

RDMC: average residual dry matter in the control.

Determination of organic matter digestibility

The DM content of feeds and feces samples was determined by oven-drying at 105°C for 48 h (AOAC, 1990; method 930.15). Ash content of samples was determined after 5h of incineration at 500°C in a muffle furnace, and the organic matter (OM) content was calculated as the difference between 100 and the percentage of ash (AOAC, 1990; method 942.05).

The dry residues were ignited in a muffle furnace at 550 °C for 5 h. The residue obtained after incineration represents the remaining mineral material. The non-degradable organic matter rate corresponding to the difference between the actual dry matter digestibility and mineral matter remaining.

DOM (%) = IOM - (IROM - ROMC) * 100 / IOM

Where: D: Digestibility of organic matter

IOM: (introduced) initial organic matter.

IROM: incubated residual organic matter.

ROMC: average of the residual organic matter in the control.

Statistical analysis

Data were analyzed by one-way analysis of variance. The differences among means for the all treatments were tested using Dunnett. Calculations was made by STATISTICA program version 6.

RESULTS

Influence of the essential oils on the in vitro gas production kinetic

Essential oil of mentha puelgium

Whatever the dose used of *Mentha puelgium* essential oils (MP-EO), the GP were decreased with the addition of the EO in all times. The magnitude of this decrease is dose dependant and it is more significant for the dose of 66.6 %L (TABLE 1). The addition of 16.6, 33.3 and 66.6 µL of EO decreased the 72 h GP to 127.33, 125.67 and 110.00 mL respectively in comparison with the control (144.33 mL).

Essential oil of thymus algeriensis

In general, whatever the dose used of *Thymus algeriensis* essential oils (TA-EO), the GP were decreased with the addition of the EO at all times. However, this decrease is more significant with the highest dose (66.6 %L) (TABLE 2). The addition of 16.6, 33.3 and 66.6 µL of EO decreased the 72 h GP to 140.67, 135.00 and 116.33 mL respectively in comparison with the control (144.33 mL).

Influence of the essential oils on the methane production

Essential oil of mentha puelgium

TABLE 1 : Effect of MP-EO addition on the methane production kinetic (ml) ± S.E.M

Dose of EO	Time after incubation (hours)					
	2	4	8	24	48	72
0µl	$7.67^{a} \pm 2.52$	$13.33^{a} \pm 3.16$	$21.00^{a} \pm 3.61$	$28.67^{a} \pm 2.52$	$32.00^{a} \pm 3.46$	$33.00^{a} \pm 3.46$
16.6 µl	$5.67^{a} \pm 0.58$	$14.33^{a} \pm 1.15$	$34.33^{b} \pm 8.08$	$45.00^{b} \pm 7.81$	$48.33^{b} \pm 8.39$	$48.67^{b} \pm 8.39$
33.3 µl	$5.67^{a} \pm 2.08$	$11.00^{a} \pm 2.00$	$16.00^{a} \pm 3.00$	$25.00^{a} \pm 3.00$	$28.33^{a} \pm 4.04$	$29.00^{a} \pm 4.58$
66.6 µl	$5.67^{a} \pm 2.01$	$8.33^{b} \pm 2.04$	$11.67^{c} \pm 2.30$	$21.00^{c} \pm 2.50$	$23.67^{c} \pm 3.06$	$25.37^{c} \pm 3.14$

a, b, c: column means without a common superscript letter differ significantly ($p < 0.05$). S.E.M.: standard error mean

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Methane concentration was decreased at highest dose of MP-EO at all times compared to the control (TABLE 1). At 72 h after incubation, the MP-EO added with 33.3 and 6.66µL reduces methane concentration at 29 and 25.37 mL respectively compared to the control (33 mL) suggesting that MP-EO is a good methane inhibitor.

However, the MP-EO used at low dose (16.6%L), increases the methane production at all times. This increase is significant after 8 h incubation and more. Using 16,6 µL of MP-EO, the amount of methane is increased at 48.64 mL after 72 h incubation (TABLE 1).

Essential oil of thymus algeriensis

Similarly, methane concentration was decreased using high doses of TA-EO at all times compared to the control (TABLE 2). At 72 h after incubation, the TA-EO added at 3.33 and 6.66µL reduces methane concentration to 29 and 25.37 mL respectively compared to the control (33 mL) suggesting that TA-EO is a good methane inhibitor.

However, the TA-EO used at low dose (16.6%L), increases the methane production at all times. This increase is significant after 8 h incubation and more. Using 16,6 µL of TA-EO, the amount of methane is increased at 48.64 mL after 72 h incubation (TABLE 2).

Digestibility of the dry matter

Whatever the EO used, there is an improvement of the *in vitro* DM digestibility but this increase is not significant ($p>0.05$). Indeed, using MP-EO, the *in vitro* DM digestibility was increased to 69.16% compared to the control (50.57%) whereas the use of TA-EO is accompanied by an increase to only 54.55% (Figure 01).

Digestibility of organic matter

Our results showed that the use of EO is accompanied by a deterioration of the *in vitro* OM digestibility but this effect is not significant ($p>0.05$). Indeed, the *in vitro* OM digestibility recorded in the control was 74.50% whereas the use MP-EO and TA-EO decrease it to 62.05% and to 64.95% respectively (Figure 02).

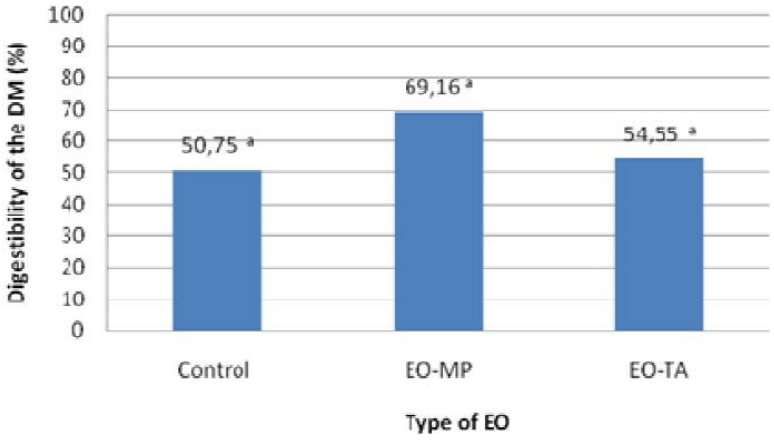
Effect of the EO on protozoa counting

The effect of the MP-EO on the ruminal protozoa

TABLE 2 : Effect of TA-EO on the methane production kinetic (ml) ± S.E.M.

Dose of EO	Time after incubation (hours)					
	2	4	8	24	48	72
0µl	$7.67^{a} \pm 2.52$	$13.33^{a} \pm 3.16$	$21.00^{a} \pm 3.61$	$28.67^{a} \pm 2.52$	$32.00^{a} \pm 3.46$	$33.00^{a} \pm 3.46$
16.6 µl	$8.67^{a} \pm 5.03$	$15.33^{a} \pm 6.51$	$22.67^{a} \pm 5.51$	$47.67^{b} \pm 6.35$	$51.67^{b} \pm 6.35$	$52.00^{b} \pm 6.93$
33.3 µl	$5.33^{a} \pm 2.89$	$10.33^{a} \pm 4.16$	$13.67^{b} \pm 5.13$	$25.00^{a} \pm 6.03$	$30.33^{a} \pm 4.04$	$30.33^{a} \pm 3.61$
66.6 µl	$5.67^{a} \pm 2.31$	$9.67^{b} \pm 4.04$	$13.67^{b} \pm 5.13^{a}$	$20.33^{c} \pm 10.15$	$23.67^{c} \pm 10.20$	$24.00^{c} \pm 10.20$

a, b, c: column means without a common superscript letter differ significantly ($p < 0.05$). S.E.M.: standard error mean



a: values with common superscript letter do not differ ($p > 0.05$). EO-MP : Essential Oils extracted from Mentha puelgium, EO-TA : Essential Oils extracted from Thymus algeriensis

Figure 1 : Effect of the EO on the dry matter digestibility

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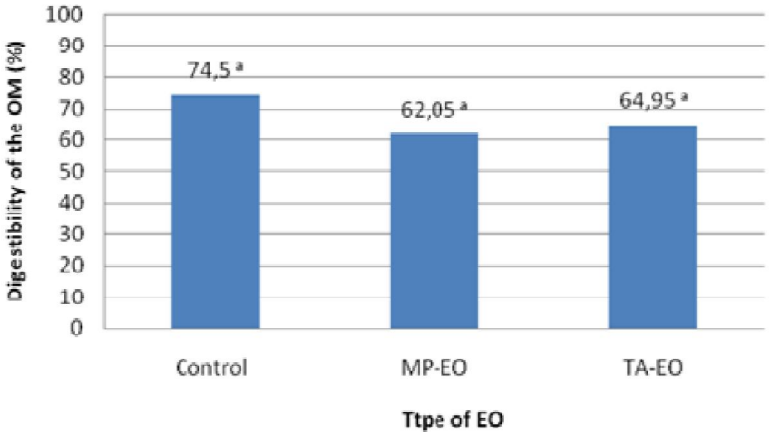

[a]: values with common superscript letter do not differ (p > 0.05), MP-EO : Essential Oils extracted from Mentha puelgium, TA-EO : Essential Oils extracted from Thymus algeriensis

Figure 2 : Effect of the EO on the organic matter digestibility

counts is presented in Figure 03. According to our results the MP-EO reduce significantly the protozoa number whatever the dose used. Indeed, the use of 0.16, 3.33 and 6.66 µl of MP-EO reduce the protozoa count to 411333 cells/ml, 432333 cells/ml and 276667 cells/ml respectively in comparison to the control (917667 cells/ml).

Similarly, the TA-EO reduce significantly the protozoa number whatever the dose used (Figure 04). Indeed, the use of 0.16, 3.33 and 6.66 µl of TA-EO reduce the protozoa count to 464000 cells/ml, 675667 cells/ml and 432667 cells/ml respectively in comparison to the control (917667 cells/ml).

DISCUSSION

A number of studies have recently evaluated the ability of essential oils to reduce enteric methane production[8, 16, 20, 24, 29, 40, 44]. In these studies, various essential oils have been tested to decrease methanogenesis. In the current study, the use of MP-EO and TA-EO resulted in a significant decrease of both total gas and methane production especially with highest doses. These results are consistent with those reported in several studies. Durmic et al. (2014) have reported a significant reduction ($P < 0.05$) in methane production was observed with eight essential oils (up to 75% reduction). Origanum and clove oils resulted in lower methane emission compared to control and peppermint oil as reported in Patra and Yu (2014). Similarly, it was found in Zmora et al. (2012) that after 24 hours of incubations, addition of 16.34 and 23.35 mg of *Mentha piperita* L. to the 233.3 mg of substrate significantly decreased methane emission by 41.52 and 15.51%, respectively. Results reported by Jahani -Azizabadi et al. (2011) indicated that coriander, cinnamon, red basil, oregano 2, cumin, caraway and dill essential oils caused a significant decrease ($P < 0.05$) in total methane production (1.5, 0.3, 1.0, 1.3, 1.1, 1.1 and 2.0 compared with 2.3 in control as mmol/g DM incubated, respectively). Finely, in a meta-analysis study conducted in different ruminant species, Khiaosa-ard and Zebeli (2013) have found that the effects of essential oils and their bioactive compounds at doses <0.75 g/kg diet DM acted as a potential methane inhibitor in the rumen as a result of decreased acetate to propionate ratio.

In this study we have use the EO of *Mentha puelgium* and those of *Thymus algeriensis* which have not been studied for this purpose before in our knowledge. Many studies reported that the effects of EO differ depending on their chemical components. Several studies on MP-EO composition have been already published, revealing a great variability in its chemical profile. Teixeira et al., (2012) showed that MP-EO revealed menthone, pulegone and neo-menthol as the main constituents (35.9, 23.2 and 9.2% respectively) while Cherrat et al. (2014) found that pulegone is the main component of MP-EO (33.65%) followed by α-pinene (24.29%) and cineole (10.53%). In general, several studies agree that MP-EO belong to "pulegone chemotype"[1, 15, 27, 43]. Likewise, TA-EO shows a great chemical polymorphism even in samples collected from the same locality[23]. Ben El Hadj Ali et al. (2012)

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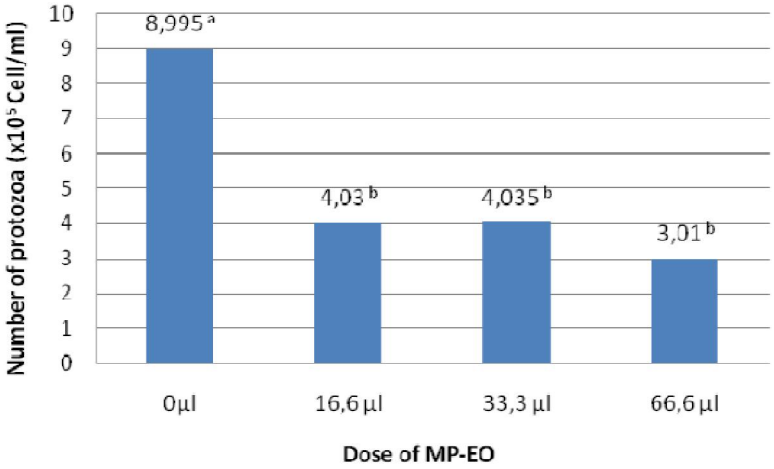

Figure 03: Effect of the MP-EO on the protozoa number, [a, b]: values with common superscript letter do not differ (p > 0.05), MP-EO : Essential Oils extracted from mentha puelgium

reported than TA-EO contains caryophyllene oxide, 1,8-cineole, α-pinene, camphor, linalool and thymol while Nikoli´c et al. (2014) revealed that thymol is the major component of TA-EO (56%). Generally, it appears that TA-EO belong to the "thymol chemotype".

The literature suggests that essential oils mitigate methanogenesis mainly by a direct toxic effect on methanogens[9, 16]. It has been suggested that essential oils cause changes in the archaeal communities, or in the activity of methanogens, to reduce the rate of CH_4 production by rumen archaea[37]. Several studies, most of them *in vitro*, have been published on effects of essential oils components on rumen microbial fermentation[8, 12]. Many of the component secondary metabolites in essential oils (Thymol, Carvacrol, Eugenol, Cinnamaldehyde…) exhibit different antimicrobial, antifungal and antiprotozoal activity[2, 9, 11, 13, 8, 17, 22, 26 29] witch influence directly the methane production. Phenolic compounds are the main active components[11, 18] although antibacterial activity has been also reported from a variety of nonphenolic substances[11, 34]. Oxygenated monoterpenes, particularly monoterpene alcohols and aldehydes, strongly inhibit growth and metabolism of rumen microorganisms[8]. The antimicrobial action of EO is due to their potential to be intruded into the bacterial cell membrane and disintegrate its structures which causes ion leakage. Essential oils have a high affinity for microbial cell membranes their lipophilic nature. The functional groups of essential oils interact with membrane components[25] and the normal ion transport across the membrane is altered. The membrane becomes disrupted and microbial enzymes are inactivated[8, 38] which compromise viability and activity of the ruminal microorganisms.

Among all rumen microbes, protozoa have a prominent position, which is strengthened by their close physical association with methanogens, which favours H_2 transfer from one to the other. A strong positive interaction was found between protozoal numbers and methane emissions[32]. In the present study, protozoa counts were reduced by approximately 30 to 50% depending on doses and type of EO compared to the control. In the literature, it appears clearly that the effect of essential oils on rumen protozoa varies. Some studies report no effect on protozoal numbers[6, 7, 30, 33] while others have found a stimulatory effect of essential oils on protozoa[2, 13, 21, 39, 42, 2] have shown that feeding 200 g/d of dried peppermint (*Mentha piperita* L.) to cannulated steers significantly decreased protozoa numbers by approximately 50%. Sallam et al. (2011) found that highest levels of *Achillea santolina*, *Artemisia judaica*, and *Mentha microphylla* EO produce a significant reduction in protozoa count. Rasmussem et al. (2005) screened 19 essential oils derived from common culinary spices and reported that the essential oil extracted from rosemary (*Rosmarinus officinalis*) had the most notable effect on protozoa. At relatively high concentrations (1000, 10000 and 40000 mg/l) they observed a reduction in protozoal viability of 50, 90 and 90% respectively (Rasmussem et al., (2005) in Hart et al., (2008)). However, Benchaar et al. (2003) reported that there

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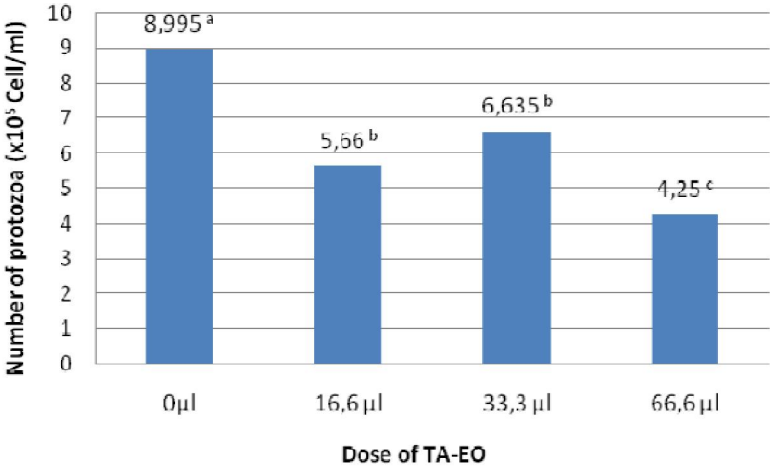

a, b, c: values with common superscript letter do not differ (p > 0.05),TA-EO : Essential Oils extracted from Thymus algeriensis

Figure 4 : Effect of the TA-EO on the protozoa number

was no effect of feeding 750 mg/d of blends EO to dairy cattle on protozoal numbers determined *in vivo*. The some observations have been reported by McIntosh et al. (2003) *in vitro*. Similarly, Newbold et al. (2004) found that there was no effect of feeding 100 mg/d BEO (circa 2.9 mg/l rumen fluid) on the number of ruminal protozoa in cannulated sheep at either 2 or 6 h post feeding. Although the mechanism of action has not been clearly elucidated, it may be related to the lipophilic nature of compounds such as anethol which facilitates permeation of essential oil across the protozoal membrane[13].

Research dealing with effects of essential oils on fungi is limited but, in general, they suggest inhibition of fungal growth[10]. The mode of action differs for each essential oil but, in general, all contain chemical constituents and functional groups, such as terpenoids, phenolic and phenols, with strong antimicrobial activity.

Clearly essential oils are able to manipulate rumen fermentation. The reported effects are likely to be due to selective pressures exerted on different microbial populations, resulting in different bacterial numbers and subsequently different activities which influence directly the nutrient digestibility. Results of this study have shown no effect of both MP-EO and TA-EO on the DM or OM digestibility. This finding is in agreement with many other studies. Indeed, Zomora et al. (2012) found that *Mentha piperita* supplementation exerted no effect on the dry matter digestibility. after 24 hours of incubations. However, Sallam et al. (2012) reported that highest levels of *Achillea santolina*, *Artemisia judaica*, and *Mentha microphylla* EO produce a significant reduction in true degradation of dry matter and organic matter. Results of the current study are very interesting because they show that EO decrease favourably CH_4 production without reducing digestibility of the dry end organic matter. However a reduction in the digestibility even if accompanied by reductions in CH_4 production, would generally be viewed to be nutritionally unfavourable, and can be associated with nonspecific depressed ruminal fermentation with less efficient rumen fermentation.

CONCLUSION

It can be concluded from the present results that both MP-EO and TA-EO significantly influenced total gas and methane production without without reducing digestibility of the dry end organic matter and with a strong toxicity towards the ruminal protozoa. The antimicrobial effect of the EO components is at the origin of this effect. The variable effect between MP-EO and TA-EO could be explained by the difference in chemical composition between the two EO.

Finally, midfielder ruminal being a complex ecosystem that brings into play different settings interfering with each other, and whose determination is essential for the understanding of many phenomena taking place there our work must be completed by:

- Further *in vitro* and in vivo trials are required to search optimum dose which reduce methane production without adversely changing dietary fermentation and rumen function.
- A determination quantitative and qualitative of

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AGV products during fermentation.

- A study *in v*ivo, which would give a closest idea to reality on methane production and food digestibility.

Toxicity to ruminants, users and consumers, palatability and effects on organoleptic quality of animal products require further research to ensure that these EO can be safely used in livestock production which allow us to advise farmers on using plants rich on essential oils in the diet of ruminants in order to combat the emission of CH_4, performs the ruminants productivity and preserve the environment.

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