Potential of eucalyptus (Eucalyptus camaldulensis) essential oil to modify in vitro rumen fermentation in sheep

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ABSTRACT

This study aimed to evaluate the effects of increasing doses (0, 10, 20, 40, 80 and 120 µl/50 ml) of essential oil of Eucalyptus (Eucalyptus camaldulensis) on in vitro rumen fermentation. Two 24 h-incubations were done and gas production (GP) was measured. The liquid was collected for analysis of ammonia nitrogen (NH₃-N) and true organic matter degradability (TOMD). The partitioning factor (PF) was estimated after 24 h of incubation. Procedure of H-NMR spectroscopy was used to analyse rumen fluid samples. Results showed that, GP was only affected and decreased significantly for the doses 20, 40, 80 and 120 µl. Ammonia-N concentration was reduced by 8.2%, 18.6%, 21.6% and 25% when 20, 40, 80 and 120 µl of EuEO were added respectively. The TOMD values were decreased significantly at doses 40, 80 and 120 µl. The PF values were affected between doses control and 120 µl. Concerning H-NMR spectroscopy, results showed that the diet containing 80 µl of EuEO, there were increases observed in the concentration of rumen methanaline, glucose, butyrate, propionate and glycine. EuEO modified fermentation trends in the rumen, mainly by reducing GP and protein deamination. And the use of EuEO in high concentrations inhibits ruminal flora and decreases the production of VFA.

Introduction

For many years, antibiotics have been used as growth promoters for livestock (Corpet 1996). In 2001, and according to the World Health Organization, this use was estimated to 50% of the worldwide produced antibiotics. However, these substances seemed to have favoured the emergence of a large number of resistant bacterial strains and allergic reactions for consumers (Corpet 1996). In 2006, the use of antibiotics to improve growth and animal performances was prohibited in the European Union (Regulation 1831/2003/EC). This led to the reappearance of pathogens responsible of causing diseases and economic losses (Alloui 2011). Consequently, considerable efforts were deployed to develop alternatives to substitute antibiotics. Among these alternatives, essential oils (EOs) are receiving a growing interest as natural antibiotic substitutes and also as beneficial additives for the manipulation of rumen fermentations (Wallace 2004). In this connection, the Eucalyptus is a native tree widely available in Tunisia, with several varieties that could provide relatively important amounts of EOs. The effects of these EOs are related to their active components and mainly chemo-types. According to Akin et al. (2010), the major components of Eucalyptus camaldulensis were ethanone (13.73%), eucalyptol (25.36%) and Caryophyllene (11.55%). Most mechanisms of action of EuEO are attributed to the interaction of oil components with the cell membrane (Benchaar et al. 2008). By another hand, the literature reported some data which had shown the potential of EOs from Eucalyptus to favourably affect rumen metabolism (Sallem et al. 2009; Patra and Yu 2012) for Eucalyptus globules. Some reported studies carried out on ruminant have shown that EuEO improved the efficiency of nutrient use by decreasing the N-NH₃ concentrations at 200 g/hd/d supplementation, tested on Holstein Friesian cows (Manh et al. 2012). Furthermore, total VFA concentrations, proportion of acetate and acetate to propionate ratio were reduced by addition of EuEO, while proportion of propionate increased (Manh et al. 2012). In the Tunisian knowledge, effects of EuEO from local ecotypes on ruminant digestion had never been investigated and the current study aimed to evaluate the impact of EOs extracted from Eucalyptus camaldulensis, on in vitro fermentation parameters in sheep.

Material and methods

Plant material

EOs was extracted by hydro-distillation (Peyron 1992) from Eucalyptus camaldulensis brought from the region of Bizerta (North West of Tunisia, humid, 600 mm of rainfall). The studied diet D was composed of 50% of ryegrass hay and 50% of commercial concentrate on dry matter (DM) basis, mixed and ground through a 1 mm screen.

Animal and rumen content sampling

Rumen content for inoculum preparation was collected from four adult male Barbarine sheep (age and live weight averaged...
12 months and 30 kg) slaughtered at the municipal slaughterhouse of Ariana municipality (Tunis). The data relative to sheep were checked from one owner and for all the incubations we used animals receiving generally oat hay supplemented with barely grain in order to standardize rumen fluid. The rumen content of the four slaughtered sheep was collected immediately after evaporation in preheated thermos (39°C) and transferred rapidly to the laboratory where the contents were mixed and filtered through four layers of surgical gas.

**Experimental design, measurements and sampling**

We measured the effect of growing doses (0, 10, 20, 40, 80 and 120 µl) of EO on in vitro rumen fermentation parameters. The medium of incubation consisted on ruminal liquid, mixed in equal proportions with a buffer solution introduced in 100 ml glass syringes (39°C) as described by Spanghero et al. (2007) and based on the method of Menke and Steingass (1988). Each dose of EuEO (five replications, through two successive incubations) was dissolved in methanol and added immediately before incubation to 0.5 g of experimental diet D. During the incubation, gas production (GP) was measured (after 2, 4, 6, 8, 10, 12 and 24 h) and pH was determined after 24 h. At the end of the incubation, the fluid samples from two syringes corresponding to each dose, were collected and conserved for ammonia-N (NH3-N) analysis (18 ml of liquid + 2 ml of conserving solution: 1% HgCl2 p/v and 5% H3PO4 v/v) and the three other syringes were used to determine the true organic matter degradability (TOMD) and to calculate the partitioning factor (PF) as described by Blümmel et al. (1997). In this procedure, it has undergone repeated washings with 100 ml of neutral detergent solution heated to reflux for 1 h and filtered with hot water (Van Soest and Robertson (1985)). The crucibles are dried in the oven overnight and weighed and then calcined at 550°C for about 6 h and reweighed after cooling in a dryer to determine the TOMD.

**Chemical analysis**

Feeds were analysed for DM according to the method No. 27.005, for ash according to the method No. 990.03 (AOAC 1984), Cell wall fractions (NDF, ADF and ADL) in feeds were determined as described by Van Soest et al. (1991). Ammonia-N was analysed according to the method of Conway (1962).

Procedure of H-NMR (Hydrogen-nuclear magnetic resonance) spectroscopy was used to analyse rumen fluid samples. Rumen samples were centrifuged to remove macromolecules (fine feed particles and microbiota). The supernatant was collected and passed through 0.2 µM syringe filters (Fischer Scientific, Fairlawn, NJ). The supernatant was collected and passed through 0.2 µM syringe filters after centrifugation (Fischer Scientific, Fairlawn, NJ). Subsequently, mix 400 µl of liquid rumen and 200 µl of a standard buffer solution (1.4425 g Na2HPO4, 0.2625 g NaH2PO4, 0.01 g NaN3 and 10 ml D2O). Immediately samples were transferred to a standard Shigemi microcell NMR tube for subsequent NMR spectral analysis (Ernst et al. 1990) to measure the effect of growing doses on rumen metabolite patterns in sheep. And for the VFA we used a multivariate analysis based on MetaboAnalyst software.

**Calculations and statistical analysis**

The numerical data were analysed statistically according to the General Linear Model procedure (GLM) of SAS (2009) with the option of MEANS multiple ranges. The model included effects of dose, incubation and interaction. Measurements from the control syringes (T: containing only buffered solution with inoculum) were used as co-variable in order to control rumen liquid origin variation.

For multivariate analysis of rumen fluid corresponding to different doses, data were imported into the MetaboAnalyst software (http://www.metaboanalyst.ca; Xia et al. 2009) designed to permit comprehensive metabolomic data analysis, visualization and interpretation for the results of VFA.

**Results and discussion**

**Chemical composition**

The chemical composition of feeds is presented in Table 1. DM content of Eucalyptus was around 63%. Eucalyptus is relatively low in CP (6.1%). This value is lower than those obtained by Al-Masri (2003) and Manh et al. (2012), who found 9.4% and 9.5% DM, respectively. The total cell wall content (NDF) of Eucalyptus was similar to results of Al-Masri (2003) (49%) but higher than those of Manh et al. (2012) who found 34.3% DM. Eucalyptus cell wall is highly ligninified and the value of ADF reached 37.1% DM. Which was close to the value (29.8% DM) reported by Al-Masri (2003) and higher than that (22% MS) found by Manh et al. (2012).

**Rumen fermentation parameters**

The effect of different levels of EuEO on GP parameters is presented in Table 2. The results showed that after 24 h of incubation, the increase of EuEO doses resulted in a significant (P < .0001) decrease of GP by 7.4 (at 20 µl of EuEO) to 54.5% (at 120 µl of EuEO) comparatively with doses 0 and 10 µl which were equivalent. This result confirmed the trends observed by Patra and Yu (2012) who studied the effect of growing doses of EO from *Eucalyptus globulus* on in vitro GP. Busquet et al. (2005) indicated that the *in vitro* GP decreases with the increase of the doses of EuEO. This decrease can be explained by the antibacterial effect exerted by EuEO, inhibiting the growth of some bacterial (Bachir Raho and Benali 2012) and protozoal (Sallem et al. 2009) populations.
After 24 h of incubation, the effect of EuEO on pH (Table 2) was significant ($P < .0001$) only for the highest doses (80 and 120 µl). Indeed, pH values were equivalent from the dose 0 to 40 µl (averaged 6.35) and increased in 80 and 120 µl (averaged 6.44). It is worthy to note that for all the doses, the pH values remains favourable for normal rumen fermentations (Kumar et al. 2012). Our results confirmed those of Kumar et al. (2012), who noted an increase of pH by about 2.2 and 1.7% when they added 4 mg of eucalyptus powder to a moderate and high fibre content diets. Similarly, Patra and Yu (2012) found that the addition of 0.5 and 1 g/l of EuEO resulted in increased pH values, comparatively with the control. This increase is related to the antimicrobial properties of EuEO. Indeed, according to Castillejos et al. (2006), the increase of pH is associated to a significant reduction in total volatile fatty acid production, reflecting a decline in substrate fermentation because of the antimicrobial effect of EuEO compounds (Fraser et al. 2007).

Ammonia-N concentration (Table 2) increased significantly ($P < .0001$) at 10 µl of EuEO, comparatively with the dose of 0 µl (48.2 and 45.9 mg/100 ml, respectively). Thereafter, NH$_3$-N decreased to 42.1 mg/100 ml for 20 µl and then to a mean value of 35.9 mg/100 ml for 40, 80 and 120 µl of EuEO. A similar trend was observed by Sallam et al. (2009) for EuEO effect on NH$_3$-N. Also, the use of Eucalyptus powder instead of EuEO resulted in equivalent observations (Kumar et al. 2012). In contrast, when higher doses were used, Gunal et al. (2014) found that the addition of 125, 250 and 500 mg/ml of EuEO increased the concentration of NH$_3$-N by about 29%, 24% and 17%, respectively. The NH$_3$-N concentration in the rumen can increase or decrease according to the amount of deaminated protein and depending on the amount and the type of dietary carbohydrate available for microbial fermentation (Russell et al. 1983). These results leads to suggest a selective positive effect of EuEO on microorganisms noted at low doses (10 µl). However, as claimed by Castillejos et al. (2005), at higher doses, most EuEO cause an inhibition of deamination and a decrease in NH$_3$-N production provided primarily by the hyper-producing ammonia bacteria.

Table 2. Effects of increasing doses of EuEO on in vitro rumen fermentation parameters.

| Dose (µl/50 ml) | 0   | 10  | 20  | 40  | 80  | 120 | SEM |
|----------------|-----|-----|-----|-----|-----|-----|-----|
| GP 24 (ml)    | 110.3$^a$ | 102.1$^a$ | 92.6$^b$ | 77.5$^c$ | 59.2$^d$ | 51.3$^d$ | 4.898 |
| pH            | 6.32$^e$ | 6.33$^e$ | 6.36$^e$ | 6.38$^e$ | 6.45$^a$ | 6.43$^a$ | 0.011 |
| NH$_3$-N (mg/100 ml) | 45.9$^i$ | 48.2$^i$ | 42.1$^i$ | 37.4$^i$ | 36$^i$ | 34.4$^i$ | 1.327 |
| TOMD (%)      | 80.1$^f$ | 79.8$^f$ | 79.9$^f$ | 72.6$^f$ | 68.7$^f$ | 67.9$^f$ | 0.0125 |
| PF (mg/ml)    | 2.93$^b$ | 3.68$^b$ | 3.81$^b$ | 4.49$^{ab}$ | 5.26$^{ab}$ | 7.43$^a$ | 0.424 |

Notes: Values with different letters in the same line are statistically different, $P < .01$. SEM: standard error of the mean; GP: gas production; TOMD: true organic matter degradability; PF: partitioning factor.

H-NMR spectral identification

Typical H-NMR spectra of rumen fluid corresponding to diets with different doses (0, 10, 20, 40, 80 and 120 µl) of EuEO are shown in Figure 1.

In this case we also aimed to investigate whether the observed metabolite changes could give further insight into the role of EuEO feeding on both the digestion and the health of the sheep. Our results clearly showed that these different diets (0, 10, 20, 40, 80 and 120 µl) could be easily distinguished on the basis of rumen metabolites. Our data demonstrated that sheep given 80 µl of EuEO had greater concentrations of propionate, glucose, ethanol, methylene, butyrate, lysine and leucine in their rumen fluid. On the other hand, sheep given 0 and 10 µl of EuEO fell into two similar clusters characterized by higher concentrations of histidine, 3-phenylpropionate and uracile. One of the most interesting observations from these analyses was a multi-fold increase in the concentrations of methylamine (MA) in the rumen of sheep receiving the highest proportions of EuEO (i.e. 80 and 120 µl). To our knowledge, this is the first report of such an association between EuEO supply and methylamine levels. Several investigators have shown that bacterial decarboxylation of amino acids such as glycine, tyrosine, and phenylalanine (Davis and De Ropp 1961; Hill and Mangan 1964) and the degradation of plant lipids, rich in choline...
(Hill and Mangan 1964; Neill et al. 1978), contribute to the production of methylated amines in ruminant animals. In addition, research conducted by Hill and Mangan (1964) indicated that an additional contributing factor in production of methylated amines is the presence of an acidic media. The importance of methylated amines to the health of sheep is
related to the fact that if they are absorbed into the blood circulation they might be degraded by semicarbazide-sensitive amine oxidases. This degradation process leads to the production of harmful metabolites such as hydrogen peroxide and formaldehyde (Yu et al. 2006). Intriguingly, Hill and Mangan (1964) and Neill et al. (1978) demonstrated that methylated amines are converted into methane by rumen microorganisms. It is well-known that a variety of factors, including the level and the nature of dietary carbohydrate fermented in the reticulo-rumen and the ratio between acetate and propionate, affect the amount of methane emitted from cattle (Johnson and Johnson 1995).

The use of EuEO in high concentrations strongly inhibits ruminal flora, decreases digestibility and production of VFAs. Generally, the desired effects are observed at low and medium doses (Benchaar et al. 2008). Patra and Yu (2012) have found that the concentration of total VFA tends to increase linearly with doses of EuEO by 5.5% and 6.8%, respectively, for medium doses (Benchaar et al. 2008). Patra and Yu (2012) demonstrated that methylated amines are converted into methane by rumen microorganisms. It is well-known that a variety of factors, including the level and the nature of dietary carbohydrate fermented in the reticulo-rumen and the ratio between acetate and propionate, affect the amount of methane emitted from cattle (Johnson and Johnson 1995).

The small change in the production of total VFAs may be beneficial if accompanied by a change in their profile, an increase in the production of propionate and the decrease in acetate production, which means a lower production of methane (Benchaar et al. 2008).

Conclusions

It can be concluded that EuEO added to a ration composed of 50% of roughages and 50% of concentrate could induce some changes in rumen fermentation as measured in vitro by reducing GP and protein deamination. At low doses, truly degraded organic matter seemed not to be drastically affected.

The changes observed on the metabolites when supplemented with higher doses of EuEO indicate that significant alterations of rumen microflora are taking place. These effects are reflected by widespread cell death as indicated by the reduced levels of some specific amino acids and by the reduced concentrations of NH3 and VFA in rumen liquid.

In vivo trials are currently carried out to investigate the effect of EuEO on intake, digestion and performances and to control the doses of EuEO affecting sheep favourably.

Authors’ contributions

RC was in charge of writing the manuscript and interpreting the study. NM, the doctoral student’s tutor, checked the manuscript and made the corrections. RC performed tests in the laboratory and participated in the acquisition of data. CD, RC made statistical analyses. All the authors have read and approved the final paper.

Disclosure statement

No potential conflict of interest was reported by the authors.

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