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Myrtle (*Myrtus communis*) essential oil effect on *in vitro* ruminal fermentation of a diet based on ray-grass and concentrate

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Abstract. The aim of the current study was to evaluate the effect of *Myrtus communis* essential oil (EO) on *in vitro* rumen fermentation of a diet based on ray-grass and concentrate. Myrtle leaves and twigs were collected from the Eastern Region of Tunisia (Zaghouan) in spring. The EO was extracted using hydro-distillation and analyzed using GC/MS. Increasing doses of EO (0 ; 5 ; 10 ; 20 ; 40 ; 80 ; 120 µL/0.5 g of substrate) were added to a ration composed of ray-grass hay and concentrate (50/50% on DM basis) and incubated in glass syringes using rumen content from sheep as inoculum simulating rumen conditions and then fermentation parameters were measured. Tow syringes were reserved to determine true organic matter degradability (TOMD). Short chain fatty acid concentration (SC-FA) and partitioning factor (PF) were calculated. GC/MS results showed that the main components of myrtle EO were α-pinene (29.5%), limonene (20.4%) and linalool (11.5%). After 24 h of fermentation, gas production (GP) decreased significantly ($P<0.0001$) from 10 to 120 µL doses (i.e. 93.4, 84.3, 70.3, 35.3 and 25.3 ml, respectively for 10, 20, 40, 80 and 120 µL). For all these EO doses, GP values were different ($P<0.0001$). The same trend ($P<0.0001$) was observed for calculated SC-FA concentrations. Concerning TOMD, no significant differences were noted between 0, 5 and 10 µL doses (averaged: 60.3%), but values decreased significantly ($P<0.0001$) for 20, 40, 80 and 120 µL (i.e. 54.9%, 49.8%, 45.9%, and 44.0% respectively). Partitioning factor values were equivalent for 0, 5 and 10µL of EO (Averaged: 6.61), but the observed values increased significantly ($P<0.0001$) for 20µL (7.12), 40µL (7.85), 80 µL (14.06) and 120 µL (18.51). It was concluded that myrtle EO can be envisaged as a potential additive to manipulate rumen fermentation and improve feed efficiency in ruminant.

Keywords. Essential oil – *Myrtus communis* – *In vitro* Fermentation – Sheep.

Effet de l'huile essentielle du myrte (**Myrtus communis*****) sur la fermentation ruminale* **in vitro** *d'une ration basée sur ray-grass et concentré***

Résumé. *Le but de cette l'étude était d'évaluer l'effet de l'huile essentielle (HE) du* Myrtus communis *sur la fermentation ruminale* in vitro *d'une ration avec ray-grass et concentré. Les feuilles et les rameaux du myrte ont été collectés à partir de la région de Zaghouan (Nord de la Tunisie, semi-aride) au printemps. L'huile essentielle (HE) a été extraite à l'aide d'hydro-distillation puis analysée par GC / MS. Des doses croissantes d'HE (0 ; 5 ; 10 ; 20 ; 40 ; 80 ; 120 µL / 0,5 g de substrat) ont été ajoutés à une ration composée de foin de ray-grass et de concentré (50:50% sur la base de MS) puis incubées dans des seringues en verre inoculées avec du jus du rumen des mouton simulant les conditions du rumen afin de mesurer les paramètres de fermentation. Deux seringues ont été réservées pour déterminer la dégradabilité réelle de la matière organique (TOMD). La concentration en d'acides gras de courte chaîne (SC-FA) et le facteur de partitionnement (PF) ont été calculés. Nos résultats (GC-MS) ont montré que les principaux composés de l'HE du myrte sont l'α-pinène (29,5%), le limonène (20,4%) et le linalol (11,5%). Après 24 h de fermentation, la production de gaz (GP) a diminué d'une façon significative (P <0,0001) de 10 à 120 µL (93,4 ; 84,3 ; 70,3 ; 35,3 et 25,3 ml, pour 10, 20, 40, 80 et 120 µL, respectivement). Pour toutes ces doses d'HE, les valeurs GP étaient différents (P <0,0001). La même tendance (P <0,0001) a été observée pour les concentrations SC-FA calculées. Concernant la TOMD, aucune différence significative n'a été notée entre les doses 0, 5 et 10 µL (en moyenne : 60,3%), mais les valeurs ont diminué significativement (P <0,0001) pour les doses 20 , 40 , 80 et 120 µL (de 54,9% ; 49,8% ; 45,9 % et 44,0% respectivement). Les valeurs du PF étaient équivalentes à 0, 5 et 10 µL de l'HE (moyenne : 6,61), mais elles* *ont augmenté de manière significative (P <0,0001) pour les doses 20 (7,12), 40 (7,85), 80 (14,06) et 120 µL (18,51). Il a été conclu que l'HE de myrte peut être envisagée comme additif permettant d'améliorer l'efficience de la fermentation ruminale.*

Mots-clés. *Huile essentielle* – Myrtus communis – *Fermentation* in vitro – *Mouton.*

I – Introduction

Antibiotics growth promoters have been successfully used to promote animal performance. However, emergence of antimicrobial resistance in humans led to their ban in the European Union since 2006. This increased the interest to evaluating soft alternatives that could modify rumen microbial fermentation. Since essential oils (EO) are known to have selective antimicrobial properties, several studies were deployed to evaluate their ruminal effects and mentioned that EO can positively impact ruminal protein metabolism, volatile fatty acid (VFA) production, fibre digestion as well as methane production (Castillejos *et al.,* 2006; Benchaar *et al.,* 2007; Ahmed *et al.,* 2014).

In this connection, myrtle is a medicinal plant endemic to the Mediterranean regions. Since antiquity, it has been used for various therapeutic and cosmetic properties. Evergreen, this shrub is widely grazed by sheep and goats in maquis. The aim of the current study was to evaluate the effect of increasing doses of myrtle EO, a frequently grazed specie in the northern region of Tunisia, on *in vitro* fermentation parameters of a diet based on ray-grass and concentrate using rumen content from sheep as inoculum.

II – Material and methods

Myrtle leaves and thin twigs (of less than 4 mm of diameter) were collected from the region of Zaghouan (North of Tunisia, semi-arid) in spring 2011. After drying at ambient air for 7 days, essential oil was extracted by hydro-distillation and characterized by GC/MS (HP-5MS capillary column). Oven temperature was programmed to rise from 50 to 240 °C at a rate of 5°C/minutes. The carrier gas was He with a flow rate of 1.2 ml/minutes and a split ratio of 60:1.

The studied diet (D) was composed of 50% of ray-grass hay and 50% of commercial ovine concentrate on dry matter (DM) basis, ground and mixed through a 1mm screen. Chemical composition of Myrtle and diet components is presented in Table 1.

Two cannulated local breed sheep (averaged age and live weight: 32 months and 45 kg) were used for *in vitro* determinations. They were housed in individual pens and received twice per day 70 g kg^{-1} $LW^{0.75}$ of a diet composed of 70% oat-vetch hay and 30% barley grains on dry matter (DM) basis.

Table 1. Chemical composition of feeds (%DM)

Feeds	DM (%)	Ash	CP	NDF	ADF	ADL	FC
Myrtle leaves and twigs	89.4	3.9	9.02	41.3	25.0	9.1	5.17
Concentrate	94.1	6.6	16.1	30.8	6.2	5.1	–
Ray-grass hay	81.6	12.5	11.3	62.7	34.1	7.2	–

The GC/MS identification of volatile compounds was made by matching their recorded mass spectra with those stored in the Wiley/NBS mass spectral library of the GC/MS data system and other published mass spectra (Adams, 2004). Determination of the percentage composition was based on peak area normalization without using correction factors.

We investigated the increasing doses of identified myrtle EO (0, 5, 10, 20, 40, 80 and 120 µL) on fermentation parameters of a diet of ray-grass and concentrate (50:50). The *in vitro* gaz production was determined in 100 ml glass syringes in triplicate twice (Menke and Steingass, 1988). For each syringe containing 0.5 g of experimental diet (D) we added the EO dose dissolved in methanol then the ruminal liquid mixed with a buffer solution (1:1). Samples were incubated at 39°C. During the incubation, gas production was measured (after 2, 4, 6, 8, 10, 12 and 24 h).

Gas production was fitted by using the non-linear model of France *et al*. (2000):

$PG = b*(1- e^{-k(t-L)})$

where: PG = Gas production at time t (ml), b = asymptotic gas production (ml), k = fractional fermentation rate (h^{-1}) and L = lag time (h).

Short chain fatty acid was estimated as described by by Getachew *et al*., (2000) on the bases of 24h GP: SCFA (mM/syringe) = 0.0239 GP - 0.0601.

At the end of the incubation, the pH fluid samples was measured and the liquid was used to determine the truly organic matter degradation (TOMD) and to calculate the partitioning factor (PF) as described by Blümmel *et al*. (1997): PF (mg/ml) = TOMD (mg) /gas volume produced at 24 h (ml).

Feeds were analyzed for dry matter (DM), ash, crude protein (CP) contents and Fat content (FC) (AOAC, 1984). Cell wall fractions (NDF, ADF and ADL) were determined as described by Van Soest et *al.* (1991).

The General Linear Model procedure (GLM) of SAS (2009) with the option of LS MEANS multiple ranges was used to analyze data. The model included effects of dose, incubation and interaction.

III – Results and discussion

1. Essential oil composition

The ten most abundant EO compounds and their classification are listed in Table 2. They represent 84.4% of EO composition.

Table 2. Main compounds of *Myrtus communis*

Compounds	Chemical classification	%
α–pinene	Monoterpene	29.5
Limonene	Monoterpene	20.5
Linalool	Alcohol	11.5
Linalyl	Acetate ester	10.0
Geranyl	Acetate ester	5.6
1.8 cineole	Penolic compound	2.3
p-cymene	Monoterpene	1.4
α-humulene	Sesquiterpene	1.3
α-terpineol	Alcohol	1.2
Neryl	Acetate ester	1.0

As it was reported by several authors (Zaouali *et al.*, 2008, Yadegarinia *et al.*, 2006 and Chalchat *et al.,* 1998), we found that the chemotype in *Myrtus communis*, is α-pinene (29.5%) specific to Tunisia. However, the main components are different from other results reported in Tunisia, Morocco and Greece since the level of limonene and linalool are higher, depending on the 1-8 cineol level (Snoussi *et al.* 2008; Viuda-Martos *et al.* 2007 and Aidi-wannes *et al.*, 2010).

2. Rumen fermentation parameters

The effect of increasing levels of myrtle EO on gas production parameters is presented in Table 2. After 24 h of fermentation, GP decreased significantly (P<0.0001) from 10 to 120 µL doses (i.e. 93.4, 84.3, 70.3, 35.3 and 25.3 ml, respectively for 10, 20, 40, 80 and 120 µL). For all these doses, GP values were statistically different (P<0.0001).

To our knowledge, this is the first work that investigated myrtle EO effect on fermentation parameters, however, a study conducted in our laboratory on an anther myrtacea (*Eucalyptus camaldelensis) showed* a significant decrease of gaz production from 20 µL (92.6 mL) to 120 µL (51.3 mL). For this same plant, Sallem *et al,* (2009) mentioned a decrease of GP from 120 mL to 55 mL for 150 µL myrtle EO dose. Therefore, we notice that myrtle EO affected GP more than Eucalyptus. This GP decrease can be explained by the antibacterial effect exerted by EOs against Gram-positive and gram-negative bacteria (Kim *et al,* 1995). Also, Benchaar *et al* (2007), working on several EOs and EO components revealed antimicrobial activity of the phenolic compounds due to the hydroxyl group.

Concerning pH, our results mentioned final pH value was not affected when EO is supplied at less than 40 µL. For higher concentration (80 and 120 µL), the pH increased significantly (6.49 and 6.51 respectively; P<0.0001). A similar effect on pH was also observed by Castillejos *et al.* (2006) who reported that 400 and 500 mg/L of thymol, a phenolic compound, increased final pH in *in vitro* batch culture. The increase in final pH was associated with a significant reduction in total volatile fatty acid (TVFA) production, due to a decrease in diet fermentability.

In the current study, SC-FA was not affected at 5 µL of myrtle EO (43.7 mg/L) but decreased significantly for each of the other levels: 10, 20, 40, 80, and 120 µL (i.e. 37.5, 53.8, 26.7, 13.9 and 9.6 mg/L, respectively). This same trend was observed by Moujahed et *al.* (2013) with Rosemary EO.

Table 3. Effects of increasing doses of Myrtle EO on *in vitro* rumen fermentation parameters of a diet based on ray-grass and concentrate

Dose (µL/50 mL)	0	5	10	20	40	80	120	ESM
GP 24(mL)****	105.9^a	104.8^a	93.4^b	84.3^c	70.3^d	35.3^e	25.3^f	1.681
pH****	6.40^b	6.39^b	6.41^b	6.43^b	6.42^b	6.49^a	6.51^a	0.024
SC-FA (mg/L)****	44.5^a	43.7^a	37.5^b	35.8^b	26.7^c	13.9^d	9.6^e	1.290

$^{a, b, c, d}$ Values with different letters in the same line are statistically different.

**** P<0.0001, SEM: Standard error of the mean.

4. Microbial activity

The myrtle EO effect on TDOM and PF is presented in table 3. At low doses (0, 10 and 20 µL), the TDOM did not change significantly (averaged 60.3%). At higher doses (40, 80 and 120 µL), a significant (P<0.0001) decrease was observed (49.8, 46.0, 44.0% respectively). In the same way, Ahmed *et al*, (2014), mentioned a slight decline in TDOM for 400 and 800 µL/kg diet of Essential oil blend (eucalyptus, cinnamon, peppermint, thyme and lemon). This decrease could be associated with a decrease in microbial protein synthesis.

The partitioning of nutrients to gas, SCFA and microbial mass was evaluated by the PF. There was a wide variation among the EO levels in PF. Slightly deceased for 10 and 20 µL of EO (7.1 mg/mL), PF increased significantly for 40, 80, 120 µL (7.8, 14.0, 18.5 mg/mL respectively). Other results on *Eucalyptus camaldelensis* reported a TOMD decrease with increasing doses of EO (from 25 to 125µL of EO), this decrease was not significant (Sallam *et al*. 2009).

Table 4. Effects of increasing doses of EO from *Myrtus communis* on TDOM and PF of a diet based on ray-grass and concentrate

Dose (µL/50 mL)	0	5	10	20	40	80	120	ESM
TDOM (%)****	60.1^{a}	61.4^{a}	59.5^{a}	55.0^{b}	49.8^{c}	46.0^{c}	44.0^{d}	0.008
PF (mg/mL)**	6,3^{d}	6,4^{d}	7,1^{cd}	7,1^{cd}	7,8^{c}	14,0^{b}	18,5^{a}	0.424

$^{a, b, c, d}$ Values with different letters in the same line are statistically different.

**** P<0.0001, ** P <0.01, SEM: Standard error of the mean.

IV – Conclusion

Our results showed Myrtle EO increasing doses caused a significant decrease in gas production and short chain fatty acid during rumen fermentation of a diet based on ray-grass and concentrate but led to a pH increase for doses superior to 40 µL. Moreover, we found that for high Myrtle EO doses, the partitioning factor was promoted. It was concluded that myrtle EO can be envisaged as a potential additive to manipulate rumen fermentation of a diet based on ray-grass and concentrate and improve feed efficiency in ruminant. *In vivo* trials are currently carried out in our laboratory to investigate the effect of EO on intake, digestion and performances.

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