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EFFECT OF *EUCALYPTUS GLOBULUS* LEAVES EXTRACTS ON *IN VITRO* RUMEN FERMENTATION, METHANOGENESIS, DEGRADABILITY AND PROTOZOA POPULATION

Amina Boussaada[1], Rabah Arhab[2], Serena Calabrò[3]♦, Raffaella Grazioli[3], Maria Ferrara[3], Nadia Musco[3], Madjid Thlidjane[1], Monica Isabella Cutrignelli[3]

[1]Department of Veterinary Medicine, Institute of Veterinary and Agricultural Sciences, Batna 1 University, 05000, Batna, Algeria
[2]Department of Natural and Life Sciences, Faculty of Exact Sciences and Natural and Life Sciences, Larbi Ben M'Hidi University, 04000, Oum El Bouaghi, Algeria
[3]Department of Veterinary Medicine and Animal Production, University of Napoli Federico II, Via F. Delpino 1, 80137, Napoli, Italy
♦Corresponding author: serena.calabro@unina.it

Abstract

The aim of the research was to evaluate the effect of three *Eucalyptus globulus* extracts rich in phenolic compounds, especially flavonoids, on rumen fermentation, methane (CH_4) production, organic matter degradability and protozoa population using an *in vitro* gas production technique. Four concentrations (0, 50, 75 and 100 mg) of three Eucalyptus extracts (ethyl acetate, n-butanol and aqueous) were added to a diet of ruminants (forage: concentrate ratio 60:40) and incubated at 39°C under anaerobiosis with buffered rumen fluid. After 24 h, the fermentation fluid was analysed for ammonia-N and volatile fatty acids (VFA). Organic matter degradability (OMD) and protozoa were also determined; *in vitro* gas production was also recorded and CH_4 concentration was measured. Compared to the control, CH_4 production was significantly lower for ethyl acetate extract ($P<0.05$), but higher for n-butanol and aqueous extracts. Production of ammonia-N was lower in all Eucalyptus extracts ($P<0.05$). Propionate production ($P<0.05$) increased for ethyl acetate and n-butanol extracts, whereas no effect was registered for VFA, for all Eucalyptus extracts. Ethyl acetate extract decreased *in vitro* OMD ($P<0.05$), whereas n-butanol and aqueous extracts were comparable to the control. Protozoa population decreased ($P<0.05$) for all extracts in comparison with the control. Eucalyptus ethyl acetate extract might be promising to be used as a potent anti-methanogenic additive. Moreover, the assessment of the right dosage seems to be important to decrease methane production, without reducing feed nutritional value.

Key words: flavonoids, gas production, volatile fatty acids, ammonia-N, partitioning factor

In livestock production systems, antibiotics are commonly used to prevent diseases and metabolic problems in animals, as well as to improve feed efficiency. A number of chemical feed additives such as antibiotics, ionophores, methane inhibi-

tors and defaunating agents have been developed to improve the efficiency of rumen fermentation by decreasing the total amount of methane or ammonia-N produced (Patra and Saxena, 2009). However, most of these additives are not used routinely because of the toxicity problems for animals, the residue issue of these chemicals in milk and meat with negative effects on human health. Furthermore, the increased use of antibiotics results in bacterial resistance; and most of them are banned in Europe since 2006 (EC Regulation No. 1831/2003). Consequently, considerable effort has been devoted towards developing alternatives to these chemical methods. Currently, numerous studies have attempted to exploit the plant secondary metabolites (i.e. plant extracts, saponins, tannins, phenolics and essential oils), which play important roles in plants, to guarantee normal growth development and defence against infection and injury. These compounds, used as natural feed additives, can improve the efficiency of rumen fermentation such as enhancing protein metabolism and decreasing methane production (McIntosh et al., 2003). Enteric methane emission is one of the most important sources of greenhouse gas and its contribution from ruminants is expected to grow as livestock production increases in the developing world to meet demand increases. *Eucalyptus* spp. is a tall native evergreen tree and it grows in many parts of the world, including Algeria, Australia, China, Egypt, India, Portugal, Spain, the southern United States, and South America (Sallam et al., 2009). The wide variety of oils extracted from Eucalyptus plant is well known in the traditional medicine for the numerous biological activities (i.e. bacteriostatic, fungistatic, anti-protozoal, anti-inflammatory) as reported by Silva et al. (2003) and Thao et al. (2015). The essential oils extracted from areal parts of the plants by steam distillation, can influence rumen fermentation due to their toxicity, modifying cell permeability, to some unfavourable strains of bacteria, such as methanogens (Thao et al., 2015). Among Eucalyptus, *E. globulus* Labill. is the species most used for industrial purposes in Europe, due to its good adaptation to the climate conditions of Mediterranean region and its fast growth (Cerasoli et al., 2016). Although this species has modest concentrations of crude oil compared with other Eucalyptus, the large amount of leaf biomass helps to increase the production per unit land area. Eucalyptol (1,8-cineole) is the main active ingredient in Eucalyptus oil from *E. globulus*. Recently, many reviews have been published on the effect of essential oils of many species of *Eucalyptus* spp. (Sallam et al., 2010; Sobhy et al., 2010; Manh et al., 2012), but there is still little experimentation on *in vitro* effects of *Eucalyptus globulus* (Ravindra et al., 2009; Ulger et al., 2017), especially using different kinds of extracts. Essential oils can be extracted from plants with various extraction methods, which can have a strong influence on their quality; an inappropriate extraction procedure can damage or alter the chemical structure of essential oil, which may lose its bioactivity and natural properties (Cetkovic et al., 2007). Essential oils can be extracted from plant materials by several methods (i.e. steam distillation, hydrodistillation, solvent extraction) in function of starting botanical material or the compounds to be separated.

The objective of this study was to evaluate the effects of three phenolic compounds-rich extracts (i.e. ethyl acetate, n-butanol, aqueous) of *Eucalyptus globulus* at three levels of inclusion on *in vitro* fermentation characteristics, rumen methanogenesis and protozoa population. It was expected to find a reduction in methane

production following the addition of Eucalyptus extracts and it was assumed that extracts may have different effects.

Material and methods

Experimental design

Three extracts (ethyl acetate, n-butanol, aqueous) of *Eucalyptus globulus* plants were tested in four dosages (0, 50, 75 and 100 mg, D0, D50, D75, D100 respectively) with the *in vitro* gas production technique (IVGPT) using as substrate a diet for ruminant and as inoculum buffered rumen liquor from cow. Two consecutive gas runs were carried out. *In vitro* fermentation characteristics (i.e. gas, degradability, end-products) were determined after 24 h of incubation, as well as methane (CH_4) production and protozoa count.

Sample collection and preparation

Samples of *Eucalyptus globulus* plants were collected during the flowering stage (April 2016) in Ain Beida, located in Oum El Bouaghi city, Algeria (Latitude 35°47′47″ North, Longitude 7°23′34″ East; 1008 m a.s.l.). The selection of this species was based on its abundance in the area of study. The aerial parts of the plant (stems, leaves and flowers) were cleaned, air-dried and ground to pass through a 1.1 mm screen (Brabender Wiley mill, OHG Duisburg, Germany), and kept in closed jars in a dry, cool place.

Extraction procedure

The extraction of the three fractions rich in flavonoids is carried out from the aerial part of *Eucalyptus globulus* plant according to the protocol of Cetkovic et al. (2007), based on the degree of solubility of the flavonoids in organic solvents. First, finely ground dry matter (450 g) was macerated with methanol/water ($MeOH/H_2O$, 70:30) three times, then a fourth maceration with hot water to renew the solvent was made. Subsequently, the recovered hydro-alcoholic extracts were collected and filtered through Büchner and then subjected to evaporation under low pressure at 35°C with a Rotavapor® (R.215. BUCHI, Labortechnik AG, Flawil, Switzerland). Thus, the obtained final residue was added to boiling distilled water and then filtered; the filtrate was then freed from waxes and lipids with hexane and chlorophyll by washing to obtain an aqueous phase. Finally, in order to separate the flavonoids of the organic phase, the remaining aqueous phase was submitted to extractions with ethyl acetate (2 × 200 mL) to separate into aglycone and mono-glycoside and with *n*-butanol (2 × 200 mL) to recover, in particular, the di- and tri-glycoside flavonoids; the final aqueous phase mainly contains the most polar glycosylated flavonoids.

Thereby, the three fractions obtained were evaporated under reduced pressure in the Rotavapor® at 45°C, lyophilized (Alpha 1-4 LD plus, Bioblock Scientific, Illkirch Cedex, France) and stored at 4°C until use.

Chemical analysis

In order to formulate a balanced diet recommended for dairy cow (forage: concentrate ratio 60:40) to use as substrate in the IVGPT trial, four ingredients (alfalfa hay, ryegrass hay-silage, solvent-extracted soya and maize) were chosen and appropriately combined (26.5, 36.5, 10.0 and 30.0%, respectively). The obtained diet was analysed for dry matter (DM), crude protein (CP), ether extract (EE) and ash, as reported by AOAC (2005) procedures (ID number: 2001.12, 978.04, 920.39 and 930.05 for DM, CP, EE and ash, respectively). Neutral detergent fibres (NDF, with sodium sulphite and heat-stable α-amylase and expressed exclusive of residual ash), acid detergent fibres (ADF, expressed exclusive of residual ash) and acid detergent lignin (ADL, determined by solubilisation of cellulose with sulphuric acid) were analysed according to Van Soest et al. (1991), using ANKOM 200 Technology (Fairport, New York, USA); hemicellulose and cellulose were estimated as the difference between NDF and ADF and between ADF and ADL, respectively. Starch content was measured after acid hydrolysis by polarimetric detection (Musco et al., 2015). The nutritive value, expressed as net energy for lactation was also calculated according to Jarrige (1998). The chemical composition of the diet is reported in Table 1.

Table 1. Chemical composition (g·kg^{-1} DM) of the diet used as substrate in the *in vitro* test

Component	Diet
Dry matter	907.5±1.0
Ash	63.8±2.1
Crude protein	115.9±2.1
Ether extract	37.1±1.5
Starch	142.9±3.2
NDF	395.1±7.1
ADF	235.9±6.6
ADL	69.1±1.3
Cellulose	166.8±5.7
Hemicellulose	159.1±2.3
Net Energy for lactation (MJ·kg^{-1} DM)	1.51

NDF: neutral detergent fibre; ADF: acid detergent fibre; ADL: acid detergent lignin; hemicellulose: NDF – ADF; cellulose: ADF – ADL.

***In vitro* fermentation characteristics**

Six dairy cows (mean body weight 680 kg) fed a total mixed ration containing corn silage, oat hay and concentrate (CP 120 and NDF 435 g·kg^{-1} DM) were used as rumen fluid donors. In particular, equal volume of material was collected from each cow at a slaughterhouse authorized according to EU legislation (R regulation (EC) No. 882/2004). The collected rumen fluids were quickly transported to the lab

in pre-heated thermos flasks, mixed, and strained through four layers of cheese cloth ensuring the temperature of 39°C and the anaerobic conditions, according to the protocol suggested by Calabrò et al. (2015). The rumen fluid (10 ml) was then mixed to anaerobic medium (75 ml) and reducing agent (4 ml) and dispensed anaerobically into 120 ml serum flasks containing 1.0051±0.022 g of substrate; treatment flasks were then supplemented by 50, 75 or 100 mg of each fraction of Eucalyptus extracts (D50, D75, D100, respectively); the substrate incubated without Eucalyptus extract represented the control (D0). The extract concentrations utilized in the present study were based on the result of a preliminary *in vitro* trial (results not presented). The serum flasks were sealed and held in an incubator at 39°C for 24 h. Three replicates for each fraction were made, and three serum flasks without extracts and substrate were incubated as blanks to correct organic matter (OM) disappearance, gas produced and end-products. Immediately after 24 h of incubation, total volume of gas (GP24) accumulated in the headspace of each serum flask was recorded as reported by Calabrò et al. (2012) using a manual pressure transducer (Cole-Parmer Instrument Co., Barrington, IL, USA). In order to measure CH_4 production the gas phase from each serum flask was sampled (3 ml) in duplicate with a gastight syringe; the analysis was carried out using a gas chromatograph (GC Trace 1310, Thermo Scientific, Waltham, MA USA) equipped with a loop TC detector and a packed column (Hay-SepQ SUPELCO, 3/16 inch, 80/100 mesh), as reported by Guglielmelli et al. (2011). The organic matter degradability (OMD, %) was determined by weight difference of the incubated OM and the undegraded residue throughout sintered glass crucibles (Schott Duran, Mainz, Germany, porosity #2).

Fermentation end-products

For volatile fatty acids (VFA) determination, a sample of fermenting liquor was centrifuged at 12000 × g for 10 min at 4°C (Universal 32R centrifuge, Hettich FurnTech Division DIY, Vlotho, Germany) and an aliquot (1 ml) of supernatant was mixed with 1 ml of oxalic acid (0.06 mol); VFA were measured by gas chromatography (GC Focus AI 3000, Thermo Scientific, Waltham, MA, USA) equipped with a fused silica capillary column (30 m × 0.25 mm i.d., 0.25 μm film thickness) as described by Musco et al. (2016).

Protozoa quantification

At 24 h the fermentation liquor (100 μL) of each serum flask was treated with methyl green formalin-saline solution (Ogimoto and Imai, 1981). The enumeration was carried out on haemocytometer (Malassez cell, 0.200 mm depth), a thick glass plate with a count chamber consisting of 100 rectangles, of which 25 subdivided into 20 small squares; the total volume of the cell was equal to 1.0 μL and 0.01 μL per rectangle. The microscopical counting was done on the rectangles subdivided into small squares using a microscope (Zeiss Binocular Microscope Axiostar, 1122-100) with a × 40 objective lens. Each sample was counted twice, in the case of differences between the two results greater than 10%. The number of protozoa is expressed according to the following equation:

$$N = n1 \times v \times n2 \times f \times 1000$$

where:

N is the number of cells per ml,
n1 is the number of cells counted,
v is the volume of a rectangle (0.01 µl),
n2 is the number of rectangles counted (25),
f is the dilution factor (Sauvant and Gouet, 1970).

Data processing

According to Blümmel et al. (1997), the partitioning factor (PF, mg/ml), an indicator of the fermentation efficiency, was calculated as ratio between *in vitro* degraded OM (OMD, g) and the volume of gas produced (G24, ml) at the end of incubation. The microbial biomass yield (MBY, mg) was also calculated using OMD, GP24 and a stoichiometric factor (2.25) as follows (Blümmel et al., 1997):

$$MBY = OMD + G24 \times 2.25$$

All the *in vitro* data on rumen fermentation characteristics and protozoa quantification were analyzed using the GLM procedure of SAS (SAS/STAT® 9.2 2014 User's Guide 2nd ed.) in a three extract (ethyl acetate, n-butanol, aqueous) by four doses (D0, D50, D75, D100) factorial design. Because extract × dose interactions were significant for most of the parameters, data were then analyzed among doses of each extract to test the dose effects. Orthogonal polynomial contrasts were used to examine the linear, quadratic, and cubic effects of the increasing doses with the same statistical procedure. The significance level was set at 0.05.

Results

The effects of the *Eucalyptus globulus* extracts on the *in vitro* fermentation characteristics are reported in Table 2. In general, the ethyl acetate extract showed the most pronounced effects. In particular, it exhibited a linear and quadratic decrease of OM degradability (%, $P<0.001$) and linear, quadratic and cubic effect on methane production (ml/g, $P<0.001$), whereas the other two extracts did not affect these two parameters. The gas produced at 24 h (ml/g) was only influenced by the n-butanol extract addition ($P<0.05$). The inclusion of ethyl acetate and n-butanol extract also decreased partitioning factor ($P<0.01$). The aqueous supplementation did not show any significant effects on *in vitro* fermentation characteristics. Comparing the extracts, significant differences ($P<0.001$) appeared only for OMD and PF (data not shown).

Table 2. Effect of *Eucalyptus globulus* extracts at different doses on the *in vitro* fermentation characteristics after 24 h of incubation

		GP24 (ml/g)	OMD (%)	CH_4 (ml/g)	PF (mg/ml)	MBY (ml)
Control	D0	102	63.0	12.2	6.21	195
Ethyl Acetate Extract	D50	109	58.6	2.00	5.38	208
	D75	113	54.5	1.14	4.86	216
	D100	121	52.2	0.79	4.32	232
MSE		149	3.49	0.41	0.25	546
Contrast	Linear	NS	***	***	**	NS
	Quadratic	NS	***	***	**	NS
	Cubic	NS	NS	***	NS	NS
n-butanol Extract	D50	110	59.2	12.3	5.39	211
	D75	133	60.0	14.7	4.56	254
	D100	139	57.1	15.4	4.18	267
MSE		317	9.93	4.97	0.324	1155
Contrast	Linear	*	NS	NS	**	*
	Quadratic	*	NS	NS	**	*
	Cubic	NS	NS	NS	NS	NS
Aqueous Extract	D50	133	64.8	15.4	4.98	254
	D75	113	66.0	13.0	5.86	216
	D100	116	60.4	14.2	5.23	222
MSE		222	8.07	2.94	0.314	823
Contrast	Linear	NS	NS	NS	NS	NS
	Quadratic	NS	NS	NS	NS	NS
	Cubic	NS	NS	NS	NS	NS

GP24: cumulative gas production related to incubated organic matter; OMD: *in vitro* organic matter degradability; CH_4: methane production related to incubated OM; PF: partitioning factor; MBY: microbial biomass yield. D0, D50, D75 and D100: 0 (control), 50, 75 and 100 mg of added extract in the culture flasks, respectively. MSE: Mean square error. *** $P<0.001$; ** $P<0.01$; * $P<0.05$; NS – not significant.

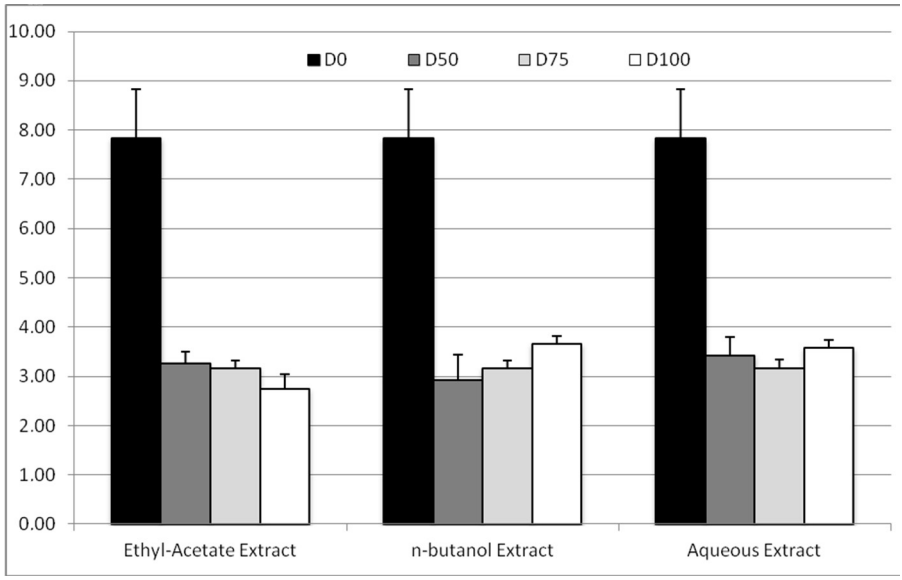
Table 3. Effect of *Eucalyptus globulus* extracts at different doses on fermentation end-products after 24 h of *in vitro* incubation

		N-NH_3 (mM/g)	tVFA (mM/g)	Acetic (mM/g)	Propionic (mM/g)	Isobutyric (mM/g)	Butyric (mM/g)	Isovaleric (mM/g)	Valeric (mM/g)	AP	BCP
Control	D0	60.0	88.4	52.0	23.7	0.623	9.09	1.21	1.69	2.21	2.07
Ethyl Acetate Extract	D50	32.2	81.6	43.4	27.4	0.377	8.55	0.517	1.48	1.59	1.10
	D75	24.2	69.7	41.9	24.8	0.340	6.30	0.390	1.15	1.69	1.04
	D100	22.7	81.8	45.8	28.1	0.357	5.66	0.493	1.37	1.63	1.04
MSE		5.67	7.40	12.1	4.83	0.001	0.332	0.0092	0.042	0.023	0.006
Contrast	Linear	***	*	NS	*	***	***	***	NS	**	***
	Quadratic	***	**	NS	NS	***	***	***	*	**	***
	Cubic	NS	**	NS	NS	NS	NS	NS	NS	NS	**
n-butanol Extract	D50	23.0	86.7	51.5	25.6	0.393	7.43	0.593	1.15	2.02	1.14
	D75	38.6	81.4	48.9	24.9	0.353	7.39	0.627	1.21	1.88	1.21
	D100	25.8	91.1	53.3	26.9	0.377	8.50	0.640	1.25	1.98	1.12
MSE		5.09	49.2	23.0	5.59	0.0013	0.197	0.0025	0.046	0.026	0.004
Contrast	Linear	***	NS	NS	NS	***	NS	***	*	NS	***
	Quadratic	***	NS	NS	NS	***	NS	***	NS	NS	***
	Cubic	*	NS	NS	NS	NS	NS	***	NS	NS	**
Aqueous Extract	D50	39.8	81.7	47.5	23.1	0.457	8.40	0.770	1.48	2.06	1.50
	D75	38.0	93.0	55.6	26.1	0.437	8.61	0.860	1.34	2.13	1.39
	D100	34.9	92.0	55.4	25.6	0.460	8.28	0.850	1.21	2.17	1.49
MSE		4.23	18.4	3.86	2.32	0.0009	0.206	0.0054	0.108	0.037	0.0041
Contrast	Linear	***	NS	NS	NS	***	NS	***	NS	NS	***
	Quadratic	***	NS	NS	NS	***	NS	**	NS	NS	***
	Cubic	NS	NS	NS	NS	NS	NS	*	NS	NS	NS

N-NH_3: ammonia-N; tVFA: total volatile fatty acids = acetate + propionate + butyrate + iso-butyrate + valerate + iso-valerate; A/P: acetate/propionate ratio; BCP: (branched-chain fatty acid proportion) = (iso-butyrate + iso-valerate) / tVFA. D0, D50, D75 and D100: 0 (control), 50, 75 and 100 mg of added extract in the culture flasks, respectively. MSE: Mean square error. *** $P<0.001$; ** $P<0.01$; * $P<0.05$; NS, not significant.

The *in vitro* fermentation end-products are reported in Table 3. Ammonia nitrogen concentration tended to decrease with linear (P<0.001) and quadratic (P<0.001) effect with increasing the dose of the three Eucalyptus extracts. The supplement of the three types of Eucalyptus extracts did not affect the main fatty acids concentration (acetic, propionic and butyric) and only the ethyl acetate extract influenced the total volatile fatty acids production with a linear (P<0.05), quadratic (P<0.01) and cubic (P<0.01) effect. Conversely, branched chain fatty acids (isobutyric and isovaleric) and their proportion in total VFA (BCP) were significantly reduced by the addition of all Eucalyptus extract and the dose response exhibited linear (P<0.001) and quadratic (P<0.001) tendency. Comparing the extracts, significant differences (P<0.001) appeared for the $N\text{-}NH_3$, branched chain fatty acids and AP and BCP ratio, showing the highest values in the aqueous extract (data not shown).

As reported in Figure 1, results of the current experiment illustrated that all extracts caused a decrease in protozoa population after 24 h of incubation: the total count of protozoa population for all *E. globulus* extracts was considerably lower than in control and the dose response had in all cases linear, quadratic and cubic effect (P<0.001). However, the effect was different depending on extract type and dose: for ethyl acetate extract the lowest protozoa count was observed in D100, whereas for n-butanol extract in D50 and for aqueous extract in D75. Comparing the extracts (data not shown), significant differences (P<0.001) appeared only with the control (D0).



D0, D50, D75 and D100: 0 (control), 50, 75 and 100 mg of added extract in the culture flasks, respectively.

Figure 1. Effect of *Eucalyptus globulus* extracts on total protozoa counts after 24 h *in vitro* incubation

Discussion

In vitro fermentation characteristics

The gas production during the *in vitro* incubation is generally a good indicator of rumen degradability, fermentability and microbial activity: higher values of gas indicate a better nutrient availability for rumen microorganisms (Makkar et al., 1997). In our study, *E. globulus* extracts increased gas production, even if the responses were different in function of the doses. Some researchers also mentioned the increase in gas production during *in vitro* incubation for other plant extracts, including Eucalyptus, rich in secondary metabolites (Jiménez-Peralta et al., 2011; Sallam et al., 2010). The increase in gas production could be ascribed to the limited negative effect of secondary metabolites, especially flavonoids, in the extracts studied. These metabolites may have positive impacts on rumen fermentation due to their low or moderate amount (Jiménez-Peralta et al., 2011; Salem et al., 2014), or to the ability of rumen microorganisms to utilize them as an energy source (Salem et al., 2014; Hart et al., 2008). Indeed, the phytochemicals, especially enzymes (i.e. amylase and lipase) present in *Eucalyptus globulus* extracts may have supported fibrolytic microbes in the rumen by increasing the proximity between substrates and microbes (Morgavi et al., 2000), favouring the stimulation of bacterial activity and consequently causing faster fermentation rate and substrate degradation (Akanmu and Hassen, 2017). In the present study, the adding of ethyl acetate and n-butanol Eucalyptus extract seems to have influenced OM degradability similarly to Sirohi et al. (2012) who observed a significant decrease of *in vitro* dry matter digestibility in comparison with the control due to the addition of different methanolic plant extract. A different trend was conversely observed *in vivo* for cattle that received *Eucalyptus camaldulensis* as supplement to rice straw (Manh et al., 2012); the authors indicated that Eucalyptus addition did not affect digestibility coefficient.

Methane production

Regarding CH_4 production, among the tested extracts, only the ethyl acetate significantly decreased CH_4 production (ml/g of incubated OM), and the percentage of reduction comparing to D0 was dose dependent (83.6, 90.7 and 93.4% for D50, D75 and D100, respectively). Chaudhary and Gupta (2013) also reported an *in vitro* reduction of CH_4 production of <15% with *Eucalyptus globulus* methanolic extract compared to the control. Eucalyptus essential oil decreased methane production and methanogenic population *in vitro* (Ravindra et al., 2009; Patra and Yu, 2012). Analogous findings have been observed *in vivo* on cows receiving 100 g/day of Eucalyptus leaf meal (Manh et al., 2012) where the authors concluded that Eucalyptus leaf reduced the emission of rumen CH_4 (estimated by VFA production) in cattle without affecting the nutrients' digestibility. As known, ethyl acetate allows the dissolution of the compounds mainly rich in hydroxyl radical, such as glycosylated flavonoids. These fractions bind to bacteria glycocalyx and probably inhibit the fixation of *Archaea* bacteria to protozoa that provide them with hydrogen. The high volume of CH_4 recorded for n-butanol and aqueous extracts, as suggested by Dey et al. (2014), could be due to the fast fermentation rate and subsequent hydrogen (H_2) production

in the rumen, which enables methanogens to convert H_2 to CH_4. Accordingly, the difference in CH_4 production among Eucalyptus extracts may be attributed to the different composition and concentration of phenolic compounds, with special reference to flavonoids. This consideration also supports previous studies (Amokrane et al., 2016), which suggested that the flavonoids potential in reducing CH_4 is related to the number and the position of hydroxyl groups and to the presence of aliphatic and glycosyl groups in their structure, as reported by Cushnie and Lamb (2011). The flavonoids antimicrobial activity appears generally through the inhibition of cytoplasmic membrane function, bacterial cell wall synthesis, or nucleic acid synthesis (Cushnie and Lamb, 2011). However, the interactions with other polyphenolic compounds present in the extracts (i.e. tannins), and their concentrations also need to be taken into account for their effects on digestibility and methanogenesis (Amokrane et al., 2016; Oskoueian et al., 2013).

Partitioning factor

In the present investigation, only ethyl acetate extracts statistically influenced the partitioning factor. This parameter (i.e., mg of DM degraded/ml of gas produced) is an indicator of fermentation efficiency; with the same amount of degradable substrate, when the partitioning factor is higher, less gas is produced and more substrate is used for VFA or microbial mass production. In this study, the PF values observed for all extracts resulted in higher than the theoretically possible maximum value (4.41 mg/ml) based on stoichiometric calculation (Blümmel et al., 1997). Makkar et al. (1997) explained these high values of PF with the capacity of tannins to form complexes with proteins, which were largely insoluble in neutral detergent solution and may contribute to the undegradable fraction. In line with our findings, Sobhy et al. (2010) reported comparable PF values for *Eucalyptus citriodora* fresh (6.1 mg/ml) leaves or residue leaves (5.7 mg/ml) incubated as substrates after essential oil extraction from eucalyptus leaves after 24 h of incubation with sheep rumen fluid. These results could suggest that Eucalyptus had a potential nutritive value, which tends to enhance microbial synthesis.

Microbial biomass yield

As this parameter is estimated on the basis of gas production and degraded dry matter and combined to PF, it appears that ethyl acetate acted on the rumen microrganisms equilibrium. As suggested by Thao et al. (2015), using direct count and roll tube technique, Eucalyptus supplementation did not affect viable bacteria and fungi zoospores; in contrast, proteolytic bacteria and protozoa were significantly decreased following the addition of Eucalyptus, as probably occurred here.

Fermentation end-products

References regarding *in vitro* production of ammonia-N by Eucalyptus extracts are scarce, however a great deal of research has been carried out to evaluate the effect of Eucalyptus essential oils on *in vitro* fermentation (Ravindra et al., 2009; Salem et al., 2014; Sirohi et al., 2012; Patra and Yu, 2012); these authors noted that essential oils of Eucalyptus decreased $N\text{-}NH_3$ production. Manh et al. (2012) have

also observed that the supplementation of cattle diet by Eucalyptus leaf decreased significantly *in vivo* ammonia in rumen fluid. A decrease of N-NH_3 concentration could be explained by a specific action of Eucalyptus extracts on hyper-ammonia producing bacteria. Extract perhaps decreased ammonia concentration due to the inhibitory effect on ammonia producing bacteria as suggested by many authors (Sallam et al., 2009; Cardozo et al., 2004). This bacterial group, despite being present in low number in the rumen, is responsible for up to 50% of deamination (Russell et al., 1991). This hypothesis is strengthened by the results reported by Santana et al. (2012), who have observed that ethanol extract of Eucalyptus showed quality as a modulator of rumen fermentation decreasing the nitrogen degradation without affecting pH values.

Considering the molar proportion of each volatile fatty acid, the results indicate that the inclusion of Eucalyptus extracts induced a decrease in acetate concentration, which is in accordance with previous *in vitro* studies on the effect of plant extracts carried out by various authors (Hristov et al., 2003; Patra et al., 2006). In parallel, as found by Busquet et al. (2006), the propionate production was increased by the addition of ethyl acetate and n-butanol extracts. It was previously reported (Amokrane et al., 2016; Mitsumori and Sun, 2008) that CH_4 production in the rumen is closely related to acetate to propionate ratio and the decreased CH_4 emission led to a higher molar proportion of propionate and lower acetate to propionate ratio.

Protozoa quantification

The decrease in the total number of protozoa found in this study was probably due to the antiprotozoal activities of the extracts secondary metabolites compounds content in the Eucalyptus extract (Ravindra et al, 2009; Patra and Yu, 2012); this action resulting in a reduction of methanogens bacteria, which live free in aqueous phase or attached to protozoa (Newbold et al., 1995). Benchaar et al. (2008) and Morgavi et al. (2010) specified that decreasing the number of H_2 producers, such as protozoa, in the rumen is an important approach to reduce CH_4 emissions. For this reason, the decrease in protozoa counts and decline in CH_4 production with addition of ethyl acetate extract might be due to the reduction in the hydrogen supply for methanogenic bacteria. However, in this study protozoa numbers decreased for all extracts, but CH_4 production was suppressed only for ethyl acetate extract. The reason for the negative relationship for n-butanol and aqueous extracts could be explained by the nature of the secondary metabolites present in each extract. Effectively, Kamra et al. (2005) and Sobhy et al. (2010) indicated that plant species and secondary plant compounds may differ in their anti-methanogen and anti-protozoal activities due to the different structure, as well as the level of inclusion. On the contrary, Goel et al. (2007) have specified that *in vitro* there is no correlation between methanogens and protozoa population and CH_4 production. Further studies are thus required to better understand the relationship between microbial population and CH_4 production in the rumen.

As hypothesized, the three different *E. globulus* extracts (ethyl acetate, n-butanol and aqueous) tested in the present investigation appear to have different effects on the *in vitro* rumen fermentation, probably due to the nature, activity and concentra-

tion of their active compounds even if it was not proved by a statistical correlation. In particular, the ethyl acetate extract, seems to be the best modulator of the *in vitro* fermentation because of its capacity to stimulate gas production with minimizing methanogenesis, and protozoa. However, since this extract also negatively affected organic matter degradability and volatile fatty acids production, various levels of the extract should be tested in order to find out a suitable dose to obtain maximum returns without adversely affecting the nutritive value. Moreover, there is a need to study the adaptation of rumen microbes and protozoa population to the antimicrobial action of these plant secondary metabolites on prolonged feeding to ruminants. The *E. globulus* extracts should be also tested *in vivo* in order to elucidate the suitable dose for improving rumen fermentation with positive effects from economic and environmental impact points of view and with minimum adverse effects on livestock production.

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