Animal, page 1 of 5 © The Animal Consortium 2011
doi:10.1017/S1751731111001947

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Degradation of terpenes and terpenoids from Mediterranean rangelands by mixed rumen bacteria *in vitro*

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(Received 19 May 2011; Accepted 9 September 2011)

This in vitro *study aimed at estimating the disappearance rates of 14 terpenes and terpenoids after 24-h incubation with mixed bacteria from caprine rumens. These compounds comprised nine monoterpene hydrocarbons (δ-3-carene, p-cymene, β-myrcene, (E)- and (Z)-β-ocimene, α-phellandrene, α-terpinene, γ-terpinene and α-terpinolene), four oxygenated monoterpenes ((E)- and (Z)-linalool oxide, 4-terpinenol, α + γ terpineol) and one sesquiterpene hydrocarbon (β-cedrene). They were individually exposed to goat rumen microflora for 24 h in 70 ml culture tubes at an input level of 0.5 ml/l. Terpenoids were the least degraded, 100% of (E)-linalool oxide, 95% of (Z)-linalool oxide, 91% of 4-terpinenol and 75% of terpineol remained intact after 24-h incubation. In contrast, α-terpinolene concentration in fermentation broth extracts was below quantification limit, thus indicating an extensive, if not complete, degradation by rumen bacteria. Only 2% of the initial amounts of α-phellandrene were recovered. The other monoterpenes and β-cedrene were partly degraded, with losses ranging from 67% for δ-3-carene to 90% for (E)-β-ocimene. The corresponding rates of disappearance were between 2.67 and 4.08 μmol/ml inoculum per day.*

Keywords: monoterpene, monoterpenoid, sesquiterpene, rumen bacteria, degradation

Implications

In the West-Mediterranean grazing systems, terpenes and terpenoids, the primary constituents of essential oils in plants of the class Magnolopsida, are ingested by small ruminants kept on rangelands, and they are partly transferred to milk and cheese. They have been associated with changes in the sensorial profile of cheeses. Quantifying their metabolism by rumen microbiota in relation with their structural properties can contribute to a better understanding of the mechanisms of their transfer from plant to animal products.

Introduction

The dairy goat livestock system in Basilicata (southern Appennine range, Italy) is typical of West-Mediterranean grazing systems, as it relies on the use of rangelands of wide botanical diversity associated with a marked seasonality in plant resources (Fedele *et al.*, 2005). Grazing plants of the class Magnolopsida, with high contents of essential oils, supply goats with mono- and sesquiterpenes, as well as monoterpenoids. Once ingested, the terpenes (TERP) or their derivatives are partly transferred from plant to milk and cheese (Fedele *et al.*, 2004; Chion *et al.*, 2010). Once in milk, some, such as α-campholenal, are susceptible to biotransformation by lactic acid bacteria (Belviso *et al.*, 2011) and are associated with changes in the sensorial profile of cheeses (Addis *et al.*, 2006). Terpenes and terpenoids transfer rates vary with grazing conditions, in possible relation with the nutritional value of grazed herbage (Fedele *et al.*, 2004), but the factors controlling these transfers are not fully identified, and information is required on the metabolic fate of these molecules in the rumen, in particular on their individual degradation rates.

In this study, nine monoterpene hydrocarbons (δ-3-carene, p-cymene, β-myrcene, (E)- and (Z)-β-ocimene, α-phellandrene, α-terpinene, γ-terpinene and α-terpinolene), one sesquiterpene hydrocarbon (β-cedrene) and four terpenoids ((E)- and (Z)-linalool oxide, 4-terpinenol, α + γ terpineol) were examined for their susceptibility to degradation by the rumen microflora of goats *in vitro*. These molecules were identified as major terpenes and terpenoids in the plants grazed by lactating goats during winter and spring in Basilicata (Fedele *et al.*, 2005). In order to evaluate the terpene degradation capacity of rumen microflora, the molecules were tested at a high input concentration.

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Material and methods

Incubation procedure

Goat care and use procedures were approved by the French Ministry of Agriculture in agreement with the French regulations for animal experimentation (guideline 19/04/1988). All terpenes and terpenoids, which were oxygenated monoterpenes, were of chromatographic grade (Roth-Sochiel Sarl, Lauterbourg, France and Extrasynthèse SA, Genay, France). Their characteristics are given in Table 1. In order to facilitate reading of this paper, the term 'terpene' will designate in the remaining text both terpenes and terpenoids. The TERP were individually tested at the concentration of 0.5 ml/l of incubation medium in 24-h incubations with mixed rumen bacteria during 3 consecutive days. On each incubation day, five culture tubes were also inoculated without the addition of terpene and used as controls (CTRL). Initial molar concentrations were similar among monoterpenes, from 2.64 to 3.27 mmol/l. The lowest value was 2.28 mmol/l for β-cedrene. Culture tubes of 70 ml, sealed with rubber/polytetrafluoroethylene (PTFE)-lined screw caps ,were used for batch incubations. The culture tubes assigned to the two treatments, CTRL and TERP, were inoculated with defaunated dual-flow fermenter contents.

Ten days before batch incubations, 1.5 l of rumen contents were taken from four goats of Saanen and French-Alpine breeds fitted with a ruminal cannula. The goats were housed in the laboratory animal facilities at Grignon (France) and fed *ad libitum* a total mixed ration composed of 340 g/kg dehydrated grinded alfalfa, 140 g/kg grass hay, 310 g/kg beet pulp silage and 210 g/kg commercial concentrate. The rumen contents were immediately pooled and stored air-tight at −20°C for 3 days in order to eliminate ciliate protozoa (Jouany *et al.*, 1988), and thus increase bacterial numbers before terpene incubations. The study was focused on mixed bacteria associated with rumen contents because most terpenes and terpenoids are poorly soluble in water (Weidenhamer *et al.*, 1993) and are supposed to adsorb onto particulate matter, that is, beyond the reach of ciliate protozoa. Further, plant terpene degradation by anaerobic protozoa has never been reported to our knowledge. The defaunating method used appeared to be the most appropriate to meet our experimental needs as it does not alter rumen microflora in a permanent way, as shown by the lack of difference between untreated and refaunated animals (Jouany *et al.*, 1988). Rumen contents were then thawed and warmed at 39°C under anaerobic conditions before being introduced into four dual-flow continuous culture fermenters (working volume of 1.1 l). The dual-flow continuous culture fermenters were run for 9 days at 39°C. At 1100 and 2300 h each day they received 15 g of a pelleted diet (orchardgrass hay 650 g/kg, maize grains 200 g/kg, soyabean meal 150 g/kg). They were continuously infused with a mineral buffer (artificial saliva) solution, whose mineral composition is detailed in Broudiscou *et al.* (1999). The artificial saliva contained 0.15 g/l HCl-Cysteine as a reducing agent. The dilution rates of particle and liquid phases were set at 0.03 and 0.06/h. The absence of protozoa in fermentation media was regularly checked by microscopic observation (Broudiscou *et al.*, 1997).

All compounds were batch incubated on each of 3 consecutive days ($n = 3$ replicates). On every day of incubation, cellobiose (0.15 mol/l), maltose (0.15 mol/l), tryptone (6.25 g/l), $(NH_4)_2SO_4$ (2.36 g/l) and $(NH_4)H_2PO_4$ (8.216 g/l) were solubilised in artificial saliva (Broudiscou *et al.*, 1999) at pH 6.8. The resulting nutrient solution met the nutritional requirements of rumen micro-organisms (Broudiscou and Jouany, 1995) by providing 0.6 mmol hexoses, 5 mg N and 1.14 mg S per tube. Contents (300 g per fermenter) were anaerobically taken at 11 : 30 from the four dual-flow continuous culture fermenters, pooled and filtered through a 250 μm mesh nylon gauze. Each culture tube was then inoculated under CO_2 atmosphere with 2 ml of nutrient solution and 8 ml of microbial inoculum. The terpenes were immediately added and the tubes were tightly sealed with rubber/PTFE-lined screw caps and were kept in a stirring

Table 1 *Characteristics of the terpenes and terpenoids tested*

Name	Formula	CAS #	MW (amu)	Structure	Density (kg/l)
δ-3-carene	$C_{10}H_{16}$	13466-78-9	136.24	2 cycles	0.87
β-cedrene	$C_{15}H_{24}$	546-28-1	204.35	3 cycles	0.93
p-cymene	$C_{10}H_{14}$	99-87-6	134.21	1 cycle	0.86
(Z)-linalool oxide	$C_{10}H_{18}O_2$	5989-33-3	170.24	1 cycle	0.90
(E)-linalool oxide		34995-77-2			
β-myrcene	$C_{10}H_{16}$	123-35-3	136.23	Linear	0.79
(E)-β-ocimene	$C_{10}H_{16}$	3779-61-1	136.24	Linear	0.79
(Z)-β-ocimene	$C_{10}H_{16}$	3338-55-4	136.24	Linear	0.79
α-phellandrene	$C_{10}H_{16}$	4221-98-1	136.24	1 cycle	0.85
α-terpinene	$C_{10}H_{16}$	99-86-5	136.24	1 cycle	0.84
γ-terpinene	$C_{10}H_{16}$	99-85-4	136.24	1 cycle	0.84
4-terpinenol	$C_{10}H_{18}O$	562-74-3	154.25	1 cycle	0.93
α + γ terpineol	$C_{10}H_{18}O$	98-55-5	154.24	1 cycle	0.93
α-terpinolene	$C_{10}H_{16}$	586-62-9	136.23	1 cycle	0.89

CAS # = chemical number; MW = molecular weight.

water bath (100 r.p.m., 39°C ± 0.5). After 24 h of incubation, they were cooled at +4°C for 1 h in order to condense the terpenes before opening.

Chemical analyses

The tube contents were processed through a three-step liquid–liquid extraction procedure derived from Rutledge (1981). Immediately before extraction, individual terpenes were added to the CTRL culture tubes in identical amounts as in terpenes culture tubes. The extraction of known amounts of terpenes from similar matrices was necessary in order for comparisons to be made without bias. The fermentation broths resulting from CTRL culture tubes were thus used as matrices in the determination of initial terpene contents in culture tubes, allowing correction of terpene disappearance rates for the actual recovery rates during liquid–liquid extraction. Each tube content was thoroughly mixed with 4 ml dichloromethane/methanol 2/1 (v/v), centrifuged at 2000×**g** for 7 min and the organic phase was collected. The extraction step was repeated once with 4 ml dichloromethane/methanol 2/1 (v/v), then with 2 ml dichloromethane. The organic extracts were pooled, washed with 4 ml NaCl-saturated water, dried by filtration through a phase-separation filter paper and the volume was adjusted to 10 ml with dichloromethane. The extracts were stored air-tight at −20°C until analysis.

We separated the terpenes using a gas chromatograph Varian GC 3400 CX equipped with a flame ionisation detector, and CP-Sil 5 CB column (25 m length, 0.25 mm internal diameter, 0.25 μm film thickness, Varian SA, Les Ulis, France) with helium N55 as the carrier gas. The oven temperature, initially at 60°C for 1 min, was raised at the rate of 10°C/min up to 160°C, then at the rate of 40°C/min up to 200°C and finally held at 200°C for 1 min. The injector and detector temperatures were set at 200°C and 300°C, respectively. The peaks were identified by their retention times, which were determined by injection of individual terpenes of chromatographic grade in dichloromethane solution. The absence of interfering peaks at the retention times of the tested compounds was checked by the analysis of blanks extracted from fermenter broths.

Statistical analyses

Chromatographic results were subjected to ANOVA using a GLM procedure (Minitab, 1998). The response variables were peak area values, expressed in arbitrary units. The fixed factors were treatment (TERP *v.* CRTL) and day. We performed mean comparisons using Fisher's least-significance-difference test.

Results

The supply of terpenes in culture tubes at levels as high as 3.27 μmol/ml was required to screen these molecules on their degradabilities by mixed rumen bacteria from dairy goats, and approximate the maximal disappearance rates possible in the rumen ecosystem. Our supply rates were within the limits of published *in vitro* assays on rumen microbial fermentation in the presence of TERP (Rutledge, 1981; Castillejos *et al*., 2006), and matched the highest experimental conditions reported *in vivo* 3 g per day in goats and sheep (Narjisse, 1982), 0.5 g per day in lambs (Estell *et al*., 2002).

Incubation data are presented in Table 2. The amounts of individual terpene recovered after 24-h incubations varied from 0% to 100% of the initial amounts. Oxygen-containing compounds were the least degraded; that is, (E)-linalool oxide remained intact, whereas 91%, 95% and 75% of the initial amounts of 4-terpinenol, (Z)-linalool oxide and α + γ terpineol were recovered at the end of the incubation. In contrast, α-terpinolene concentration in fermentation broth extracts was below quantification limit, thus indicating an extensive, if not complete, degradation by rumen bacteria. Only 2% of the initial amounts of α-phellandrene were recovered. The other monoterpenes and β-cedrene were partly degraded, with losses ranging from 67% for δ-3-carene to 90% for (E)-β-ocimene.

Table 2 *ANOVA of chromatographic results in TERP and CTRL incubations, RAR after 24-h incubations and DR of individual terpenes (μmol/ml inoculum per day)*

Terpene	CTRL (aau)	TERP (aau)	s.e.m. (aau)	P < F Inc.	RAR (%)	DR
δ-3-carene	426.2	141.5	50.4	0.057	33.2	2.67
β-cedrene	279.3	62.7	1.4	0.001	22.4	2.21
p-cymene	844.0	204.5	19.1	0.002	24.2	3.04
(E)-linalool oxide	326.3	328.7	2.1	0.50	100.7	-0.02
(Z)-linalool oxide	357.3	337.5	2.3	0.027	94.5	0.18
β-myrcene	558.5	84.2	14.5	0.002	15.1	3.08
(E)-β-ocimene	394.8	39.3	28.6	0.07	9.9	3.27
(Z)-β-ocimene	182.8	28.3	13.8	0.08	15.5	3.06
α-phellandrene	239.0	5.5	3.9	0.015	2.3	3.81
α-terpinene	156.0	33.0	29.0	0.21	21.2	3.04
γ-terpinene	692.0	115.7	23.8	0.003	16.7	3.21
4-terpinenol	846.5	773.3	111.9	0.72	91.3	0.33
α + γ terpineol	508.8	383.2	54.2	0.24	75.3	0.93
α-terpinolene	164.8	0	1.7	0.001	0.0	4.08

TERP = terpenes; CTRL = controls; RAR = relative amounts recovery; DR = disappearance rates; aau = arbitrary peak area units; s.e.m. = standard error of the mean. P < F Inc.: significance level of the differences for the treatment (TERP *v.* CTRL).

The corresponding rates of disappearance were comprised between 2.67 and 4.08 μmol/ml inoculum per day.

Discussion

In Basilicata, the plant species mostly grazed by dairy goats during spring are *Lolium perenne* L., *Poa pratensis* L., *Medicago polymorpha* L., *Medicago lupulina* L., *Asperula odorata* L. and *Geranium molle* L., which contribute approximately 10%, 6%, 13%, 8%, 12% and 8% of the diet, respectively (Fedele *et al.*, 2005). These authors reported that the plants belonging to the class Magnolopsida (formerly dicotyledons) accounted for 36.5% of the biomass ingested. The winter diet was dominated by Graminacea, mostly *L. perenne* L. and *Dactylis glomerata* L., whereas Magnolopsida accounted for only 8% of the ingested plants. Among the compounds tested, p-cymene and α-phellandrene were detected in large amounts in both diets as well as, to a lesser extent, 4-terpinenol and α-terpinolene. Linalool oxides, β-cedrene and α + γ terpineol were at their highest concentrations in the spring diet, whereas δ-3-carene, β-ocimene and α-terpinene were only found in the winter diet. The monoterpene hydrocarbons and oxygenated monoterpenes tested had various structures, from linear to bicyclic molecules, whereas the sole sesquiterpene, β-cedrene, was a tricyclic molecule.

Little research has been done on ruminal degradation of terpenes. In majority of the cases, only indirect evidence of ruminal degradation of terpenes can be deduced from pharmacokinetic analyses of 1,8-cineole plasma concentration after rumen dosing (Dziba *et al.*, 2006) or from poor correlations between ocimene and myrcene contents in pasture samples and in the corresponding milk (Bugaud *et al.*, 2001). Our incubation results are consistent with a previous *in vitro* experiment, where 10 terpenes were incubated at 2 ml/l, a concentration four times higher than in this present series (Broudiscou *et al.*, 2007). We measured similar rates of disappearance, from 1.42 to 5.24 μmol/ml inoculum per day, for six readily degradable terpenes. β-myrcene, in particular, was degraded at a rate of 4.46 μmol/ml inoculum per day. This supports our hypothesis that the terpene input concentrations in this study were high enough to support maximum terpene disappearance rates, which are therefore acceptable estimates of potential degradation of terpenes in the rumen. In addition, ranking the 14 terpenes by their relative amounts recovered was highly consistent with the results of a trial conducted under both experimentally and analytically dissimilar conditions (Malecky *et al.*, 2009), in that terpenes were incubated with mixed rumen bacteria at a concentration 167-fold lower than in this study, and they were, then, extracted from fermentation broths using a head-space solid phase micro-extraction procedure. The relative amounts recovery ranged from 0.3% for α-phellandrene to 93% for (Z)-linalool oxide and correlated well with the data of this study (Y) according to the following regression equation: $Y = X \times 0.962\ (\pm 0.084) + 6.9\ (\pm 4.0$; s.d. in parentheses, $n = 14$, adjusted $R^2 = 0.91$), in spite of completely different terpene test concentrations and extraction methods.

At present, the incubation was run with protozoa-free inocula because ciliate protoza prey on bacteria, thereby lowering their total number (Jouany *et al.*, 1988). From our observations, we attempted to estimate the maximum capacity of the goat rumen bacteria to degrade individual terpenes. From the feed input per unit of volume (three times lower in dual outflow fermenters than in the rumen) and the feed constituent degradabilities and biomass synthesis efficiencies that are comparable in both cases, one can approximate that biomass contents in our inoculums were three times lower than in the original rumen fluid. As the inoculum accounted for 0.75 of the total incubation volume, we can thus extrapolate the maximum disappearance rates of the most degradable terpenes in a 10-l goat rumen that is 11, 14, 12, 13, 13 and 17 g per day for δ-3-carene, β-cedrene, p-cymene, β-myrcene, γ-terpinene and α-terpinolene, respectively. The values calculated for (E)-β-ocimene, (Z)-β-ocimene, α-phellandrene and α-terpinene were 13, 12, 16 and 12 g per day. However, the impact of repeated exposure of rumen microorganisms to terpenes on the bioconversion of the latter remains to be assessed. Properly addressing this issue will require comparing unexposed animals with those kept on Mediterranean rangelands, and therefore to carefully adapt the experimental protocol to the local agrometeorological variability and to the subsequent terpene availabilities from the plants consumed.

In conclusion, our observations clearly differentiated the terpenes in terms of rumen microbial degradation rates *in vitro* in relation with terpene structural characteristics. Monocyclic α-terpinolene and α-phellandrene were extensively degraded, followed by linear and other monocyclic monoterpenes, then by compounds with a higher number of rings. At the opposite end of the scale, oxygen-containing compounds were poorly degraded. Consequently, dietary terpene profiles are subject to significant changes following rumen digestion.

Acknowledgements

We are grateful to J. Tessier and his team for the animal care.

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