Animal (2007), **1**: pp. 675–680 © The Animal Consortium 2007
doi: 10.1017/S1751731107730781

animal

Potential of carvacrol to modify *in vitro* rumen fermentation as compared with monensin

V. García, P. Catalá-Gregori, J. Madrid[†], F. Hernández, M. D. Megías and H. M. Andrade-Montemayor[‡]

Department of Animal Production, University of Murcia, Campus de Espinardo 30071, Murcia, Spain

(Received 5 December 2005; Accepted 15 January 2007)

The aim of this study was to assess the effect of carvacrol supplement as a dietary additive to rumen fermentors, fed a barley seed:alfalfa hay (70:30) ration and to compare its effect with monensin supplementation. The material was incubated with goat ruminal fluid and four different treatments were included: no additive (C), 7.5 mg/l monensin (M), 250 mg/l carvacrol (C250) and 500 mg/l carvacrol (C500). The addition of carvacrol reduced in vitro dry matter (DM), crude protein (CP) and neutral-detergent fibre (NDF) digestion. The effects induced by C250 on DM digestion at 72 h of incubation were comparable with those of M, whereas a greater reduction was obtained when carvacrol was supplemented at 500 mg/l concentration (68.9, 68.5 and 53.0 v. 76.1% for M, C250 and C500 v. C, respectively). The reduced CP potential degradability by supplements (51.2, 53.9 and 51.5 v. 72.8% for M, C250 and C500 v. C, respectively) was mainly caused by a reduction of the slowly degradable fraction. Volatile fatty acid (VFA) profiles determined after 48 h of incubation showed C250 increased butyrate and decreased acetate proportions, whereas M mainly stimulated propionate proportions, suggesting that the mechanism of action of carvacrol and M differs. C500 significantly reduced total VFA production. Carvacrol could be of great interest for its usage as a potential modulator of ruminal fermentation. Future research, including in vivo studies, in order to understand the factors that contribute to its antimicrobial activity and the selection of the optimal dose is required.

Keywords: carvacrol, *in vitro* degradability, monensin, ruminants

Introduction

In recent years, the use of antibiotics as growth promoters in animal nutrition has been limited in the European Union (EU) because of its relation to the increase in the number of antibiotic-resistant bacteria to drugs which are used by humans (Cancho *et al*., 2000). Consequently, new commercial additives are required that offer more safety, but also have the property of manipulating rumen fermentation. Thus, additives of vegetal origin, considered to be natural products, have been proposed to livestock producers as possible replacers of growth promoting antibiotics. The antimicrobial capacity of several natural extracts, mainly essential oils, has been demonstrated (Cowan, 1999). The antimicrobial properties of essential oils could be used to modulate rumen microbial activities (McIntosh *et al*., 2000). Some effects could be related to a decrease in the number of proteolytic rumen bacteria and to a reduction in the rate of deamination in the rumen (Losa *et al*., 2002), or to other mechanisms such as the control of ruminal methanogenesis (Broudiscou *et al*., 2000; Evans and Martin, 2000). Currently, it is necessary to evaluate the effects of active components from plant extracts on ruminal parameters, in order to confirm their usefulness as an alternative to the use of growth-promoting antibiotics in ruminants.

Carvacrol and thymol, two phenolic derivatives, are considered to be the main active components in the essential oil fraction of oregano and thyme (Sivropoulou *et al*., 1996), and to exhibit antimicrobial activities (Helander *et al*., 1998). Thus, the aim of this study was to assess the influence of carvacrol on the *in vitro* ruminal degradability of dry matter (DM), crude protein (CP) and neutral-detergent fibre (NDF) and on end-products of fermentation as volatile fatty acids (VFA), in comparison with monensin.

[†] E-mail: alimen@um.es
[‡]Present address: Goat and Ruminant Nutrition, Facultad de Ciencias Naturales, Lic. Medicina Veterinaria y Zootecnia, Universidad Autónoma de Querétaro, Campus Juriquilla.

https://doi.org/10.1017/S1751731107730781 Published online by Cambridge University Press

Material and methods

Substrate

The substrate used for the *in vitro* procedure was composed of barley seed (*Hordeum vulgare* L.) and alfalfa hay (*Medicago sativa* L.) with a concentrate to forage ratio of 70:30 on a DM basis. The substrate was ground through a 1-mm screen. DM was determined by drying at 60°C for 48 h. CP (Kjeldahl N $\times$ 6.25) and ash were analysed according to the Association of Official Analytical Chemists (1990). NDF (using method with sodium sulphite and heat-stable α-amylase), acid-detergent fibre (ADF) and acid-detergent lignin (ADL) were determined as described by Van Soest *et al.* (1991). Table 1 shows the chemical composition of the incubated ingredients.

Collection of ruminal fluid

Two goats of Murciano-Granadina breed were fed alfalfa hay *ad libitum* for 15 days, sacrificed 4 h after feeding and rumens from both animals removed immediately. The content of the rumens was squeezed through four layers of cheesecloth. A volume of 1600 ml of ruminal fluid, resulting from the mixture of both ruminal contents, was purged with deoxygenated CO_2, and placed at 39.5°C just before its use as ruminal inoculum in the *in vitro* procedure.

In vitro *degradability*

The *in vitro* procedure was conducted in a commercial incubator (Daisy II$^{200/220}$ incubator, ANKOM Technology Corp., Fairport, NY) to determine the degradability of the substrate. The incubator consists of four independent digestion jars, and this incubation instrument allows keeping the fermentation medium in continuous agitation and at a specific temperature (39.5 $\pm$ 0.5°C). Each jar was filled with the digestion medium that contained: 1584 ml of buffer solution pre-warmed at 39°C, 400 ml of the ruminal inoculum and 16 ml of a solution with the test additive diluted in ethanol or ethanol alone in the negative control group. The buffer solution was made from two other buffer solutions (A and B) in a ratio of 1:5 to obtain a final pH of 6.8 at 39°C. The A buffer solution contained: KH_2PO_4 (10 g/l), $MgSO_4.7H_2O$ (0.5 g/l), NaCl (0.5 g/l), $CaCl_2.2H_2O$ (0.1 g/l) and urea (0.5 g/l); and the B buffer solution contained: Na_2CO_3 (15.0 g/l) and $Na_2S.9H_2O$ (1.0 g/l). Four treatment groups were established (i.e. one per digestion jar): negative control, without additive (C); positive control, 7.5 mg/l monensin (M); 250 mg/l carvacrol (C250) and 500 mg/l carvacrol (C500). The doses of the evaluated additives were set to meet or exceed the minimum inhibitory concentrations (MICs) provoking antimicrobial activity based on former studies (monensin, Domescik and Martin (1999); Wang *et al.* (2004) and carvacrol, Juliano *et al.* (2000)). The reactants used were: monensin sodium salt ($C_{36}H_{61}NaO_{11}$, Fluka BioChemika, Steinheim, Germany) and 5-isopropyl-2-methylphenol (carvacrol) ($C_{10}H_{14}O$, Panreac, Barcelona, Spain).

A 0.5-g sample of substrate was directly weighed into a filter bag (F57 Filter Bags, ANKOM Technology), placing 28 bags with substrate and seven blanks (without substrate, empty bags) inside each digestion jar. Once all bags were placed into each digestion jar, which was already filled with the fermentation medium, the corresponding bags per treatment were removed for 0 h of incubation. The selected incubation times were: 0, 4, 8, 12, 24, 48 and 72 h. At each time, five filter bags per treatment were randomly removed (i.e. four bags with substrate and one blank); then the jar was purged with CO_2 and sealed. The removed bags were washed with tap water until the rinsing water was clean. Washed bags were dried at 60°C for 48 h and weighed to determine DM residues. CP, by the Kjeldahl method (N $\times$ 6.25) and NDF, using the method described by Van Soest *et al.* (1991), were determined on DM residues. *In vitro* DM, CP and NDF disappearance data were calculated from the concentration of each nutrient in the residues and the original samples after correction for amounts of DM, CP and NDF in blank bags.

Table 1 *Chemical composition of incubated ingredients*[†]

	Barley seed	Alfalfa hay
DM (g /kg)	901	906
Composition (g/kg DM)		
OM	977	859
Ash	22.6	141
CP	105	184
NDF	223	431
ADF	47.1	318
ADL	2.84	58.6

[†] DM = dry matter; OM = organic matter; CP = crude protein; NDF = neutral-detergent fibre; ADF = acid-detergent fibre; ADL = acid-detergent lignin.

Volatile fatty acids (VFA)

After 48 h of incubation, a duplicate of 50 ml of the fermentation medium was removed per treatment, and centrifuged at 3000 $\times$ **g** for 20 min. Supernatants were transferred into plastic containers, 1 ml of 50% sulphuric acid was added and they were frozen at -20°C until VFA determination. The VFA concentration was measured by capillary gas chromatography as described by Madrid *et al.* (1999). The gas chromatograph (TRACE GC Ultra, Thermo Finnigan Italia SpA, Milan, Italy) was equipped with a flame ionisation detector. The capillary column was fused silica, 30 m $\times$ 0.25 mm $\times$ 0.25 μm ID and coated with FFAP-TR as the stationary phase (Teknokroma, Spain). Standard solutions of acetic, propionic and butyric acids were prepared for calibration, using 4-methyl-*n*-valeric acid (30 mmol/l) as internal standard. Original supernatant solutions were diluted twice and internal standard was added in ratio 10:1 (diluted supernatant/internal standard). The solution was mixed and centrifuged at 7000 $\times$ **g** for 7 min at 4°C. Then, 1 μl aliquot of this solution was injected onto the column.

https://doi.org/10.1017/S1751731107730781 Published online by Cambridge University Press

Kinetic of degradability and statistical analyses
In vitro nutrient disappearance data were used to estimate degradation parameters using non-linear equations. The model used was: $p = a + b\ (1 - e^{-ct})$, described by Ørskov and McDonald (1979), where p is the *in vitro* disappearance (%) at time t, a is the soluble fraction (%), b is the slowly degradable fraction (%), c is the fractional rate at which the b fraction is degraded (per h) and $a + b$ is the potential degradability. Effective degradabilities (ED) were estimated using the equation: $ED = a + bc/(c + k)$, where k is the rumen outflow rate, taken as 0.06 per h (Sauvant *et al.*, 2003).

The effects of supplements on *in vitro* DM, CP and NDF degradability for each time, the results of VFAs at 48 h of incubation and kinetic parameters of degradability were subjected to one-way analysis of variance (Steel *et al.*, 1996), using SPSS software (Statistical Packages for the Social Sciences, 1997). The comparison between means was analysed by LSD test.

Results

In vitro *degradation*
Approximately 50% of the total DM loss occurred after 12 h of incubation in all the evaluated treatments; except for C500 that had a lower DM loss (≈40%; Table 2). All supplements reduced ($P < 0.001$) potential DM degradation. This is mainly due to a reduction ($P < 0.001$) in the slowly degradable fraction (b), but fractional degradation rates were higher for M and C250 ($P < 0.05$). Consequently, effective DM degradation only has been reduced ($P < 0.001$) by C500.

Table 3 shows the effect of monensin and carvacrol on the *in vitro* CP degradability. Only C250 and C500 reduced ($P < 0.05$) CP degradation compared with the negative control after 72 h of incubation. M, C250 and C500 diminished the potential degradability of the protein by reducing the b fraction ($P < 0.05$). In addition, only C500 exerted a reduction of the CP effective degradability of approximately five points against the C treatment ($P < 0.05$).

A reduction in NDF degradability by all supplements from 24 to 72 h of incubation was found (Table 4). The highest NDF loss occurred at 72 h in C compared with the supplemented treatments ($P < 0.001$). The NDF potential degradation and effective degradability were also lower in the treated groups in comparison with the negative control ($P < 0.01$).

The kinetic model of DM and NDF degradability obtained a R^2 mean value of 0.942 and 0.954 respectively, whereas for the CP kinetic model the R^2 reached 0.750 only.

VFA production
The average concentration of VFAs and the molar proportion of each individual acid in the *in vitro* rumen medium at 0 and 48 h of incubation are presented in Table 5. The total VFA concentration after 48 h of incubation was influenced by supplements ($P < 0.001$). The VFA values in M and C250 were higher than in C after 48 h of incubation; whereas C500 showed the lowest VFA concentration. The molar proportion of acetate, propionate and butyrate was also affected ($P < 0.001$). C250 and M reduced acetate molar proportions; whereas C500 increased the acetate molar proportion. M increased

Table 2 In vitro *dry matter (DM) degradation of substrate without or with supplementation of monensin or carvacrol at two doses (C205, 250 mg/l; C500, 500 mg/l)*

	Incubation times (h)						
	0	4	8	12	24	48	72
DM degradation (%)							
Control	26.7	36.4	44.0^{bc}	48.5^{b}	60.1^{b}	69.4^{c}	76.1^{c}
Monensin	28.6	38.7	48.9^{c}	51.1^{b}	58.3^{b}	63.7^{bc}	68.9^{b}
C250	26.6	35.3	41.7^{ab}	49.6^{b}	56.5^{b}	62.8^{b}	68.5^{b}
C500	27.4	34.6	37.0^{a}	40.5^{a}	39.9^{a}	46.7^{a}	53.0^{a}
s.e.	0.8	0.7	1.1	0.9	0.9	1.0	0.6
Significance†	NS	NS	*	**	***	***	***
		Kinetic parameters $[a + b\ (1 - e^{-ct})]$					
		a (%)	b (%)	c (h^{-1})	$(a + b)$	ED‡	
Control		27.6	49.3^{c}	0.048^{ab}	76.9^{c}	49.0^{bc}	
Monensin		29.3	37.5^{b}	0.077^{c}	66.8^{b}	50.0^{c}	
C250		26.8	40.6^{b}	0.062^{bc}	67.4^{b}	47.0^{b}	
C500		30.0	23.7^{a}	0.034^{a}	53.6^{a}	38.4^{a}	
s.e.		0.6	1.0	0.004	0.9	0.4	
Significance†		NS	***	*	***	***	

[a-c] Means in the same column with different superscript letters are significantly different.
† *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; NS = not significant.
‡ ED = effective degradability values at 0.06 per h outflow rate.

https://doi.org/10.1017/S1751731107730781 Published online by Cambridge University Press

Table 3 In vitro *crude protein (CP) degradation of substrate without or with supplementation of monensin or carvacrol at two doses (C205, 250 mg/l; C500, 500 mg/l)*

	Incubation times (h)						
	0	4	8	12	24	48	72
CP degradation (%)							
Control	31.5	36.3	38.0	36.7	41.0	50.5	64.5[b]
Monensin	33.1	34.4	41.9	45.2	44.5	43.1	55.4[ab]
C250	30.3	36.2	41.4	40.0	40.6	50.9	50.5[a]
C500	30.0	30.9	32.7	34.0	40.1	39.7	46.7[a]
s.e.	0.4	0.9	2.3	1.5	1.6	2.4	1.6
Significance†	NS	NS	NS	NS	NS	NS	*
		Kinetic parameters $[a + b\,(1 - e^{-ct})]$					
		a (%)	b (%)	c (h^{-1})	($a + b$)	*ED*‡	
Control		31.1	41.7[b]	0.016	72.8[b]	40.1[b]	
Monensin		33.6	17.6[a]	0.055	51.2[a]	41.7[b]	
C250		31.8	22.1[a]	0.052	53.9[a]	40.4[b]	
C500		29.9	21.6[a]	0.020	51.5[a]	35.0[a]	
s.e.		0.5	1.9	0.009	1.7	0.5	
Significance†		NS	*	NS	*	*	

[a-b] Means in the same column with different superscript letters are significantly different.
† $*P < 0.05$; NS = not significant.
‡ *ED* = effective degradability values at 0.06 per h outflow rate.

propionate, diminishing acetate:propionate ratio, whereas the C250 treatment increased the molar proportion of butyrate.

Discussion

In the present study, carvacrol was shown to have the potential to modify *in vitro* ruminal fermentation and to increase total VFA concentration when supplemented at 250 mg/l. Despite C250 reduced DM degradability similar to M, a greater degradability reduction was obtained when C500 was included. The effect of M on DM degradability has been reported before by other authors (e.g. Wang *et al.*, 2004). On the other hand, in a study with heifers fed diets with different forage to concentrate ratios, Molero *et al.* (2004) showed that the addition (700 mg/day) of a

Table 4 In vitro *neutral-detergent fibre (NDF) degradation of substrate without or with supplementation of monensin or carvacrol at two doses (C205, 250 mg/l; C500, 500 mg/l)*

	Incubation times (h)						
	0	4	8	12	24	48	72
NDF degradation (%)							
Control	0.000	4.67[b]	13.8[bc]	22.8[b]	28.9[c]	35.1[b]	44.8[b]
Monensin	0.589	1.88[a]	9.88[a]	13.6[a]	16.9[ab]	19.4[a]	24.9[a]
C250	0.361	8.26[c]	15.0[c]	15.8[ab]	17.9[b]	19.7[a]	24.2[a]
C500	0.962	6.92[c]	10.9[ab]	12.3[ab]	14.6[a]	18.5[a]	21.1[a]
s.e.	0.3	0.2	0.5	0.9	0.3	0.6	1.3
Significance†	NS	*	*	*	***	**	***
		Kinetic parameters $[a + b\,(1 - e^{-ct})]$					
		a (%)	b (%)	c (h^{-1})	($a + b$)	*ED*‡	
Control		0.000	43.5[b]	0.048	43.5[b]	19.2[c]	
Monensin		0.000	23.4[a]	0.058	23.4[a]	11.4[a]	
C250		0.839	21.0[a]	0.120	21.9[a]	14.1[b]	
C500		1.89	18.4[a]	0.073	20.3[a]	11.5[a]	
s.e.		0.4	0.9	0.014	1.2	0.3	
Significance†		NS	**	NS	**	**	

[a-c] Means in the same column with different superscript letters are significantly different.
† $*P < 0.05$; $**P < 0.01$; $***P < 0.001$; NS = not significant.
‡ *ED* = effective degradability values at 0.06 per h outflow rate.

https://doi.org/10.1017/S1751731107730781 Published online by Cambridge University Press

Table 5 *Volatile fatty acid (VFA) concentrations at 0 h and 48 h of incubation without or with supplementation of monensin or carvacrol at two doses (C205, 250 mg/l; C500, 500 mg/l)*

	0 h incubation	48 h incubation					
		Control	Monensin	C250	C500	s.e.	Significance†
Total VFA (mmol/l)	5.68	15.5[b]	17.5[c]	17.6[c]	7.87[a]	0.1	***
Molar proportion (%)							
Acetate	85.1	67.0[c]	64.9[b]	61.2[a]	89.0[d]	0.3	***
Propionate	9.10	21.0[c]	28.2[d]	15.6[b]	6.63[a]	0.1	***
Butyrate	5.70	11.8[c]	6.76[b]	23.0[d]	4.36[a]	0.1	***

[a-d] Means in the same row with different superscript letters are significantly different.
† ***$P < 0.001$.

blend of essential oils, in which thymol (carvacrol is a naturally occurring isomer of thymol) was one of the major components, reduced *in situ* effective DM degradability of green peas compared with control diets; while *in situ* ruminal degradability of other feeds (e.g. maize-gluten feed, lupin seeds, fish, soya-bean and sunflower meal) was not affected. Moreover, Newbold *et al.* (2004) also observed a reduction of *in situ* DM degradation of soya-bean meal after 8 and 16 h of incubation when an essential oil (mixture of thymol, guajacol and limonene) was added to the diet of sheep. However, this mix had no effect on DM degradability of rapeseed meal and hay.

In our work, CP degradation had a tendency to decrease with the supplemented treatments after 72 h of incubation. Monensin has been reported before to reduce feed protein degradation in the rumen (Van Nevel and Demeyer, 1977). In the study of Molero *et al.* (2004) the blend of the above-mentioned essential oil had little effect on protein degradation: a decreased CP degradation was only observed in three of the five vegetal protein sources in heifers fed a high concentrate diet. However, in an *in vitro* ruminal fermentation study, Busquet *et al.* (2006) showed that carvacrol resulted in a 30 to 50% reduction in ammonia N concentration; but the carvacrol concentration used (3000 mg/l) was higher than both carvacrol levels used in our study.

All additives decreased NDF degradability from 24 to 72 h of incubation. Ionophores, such as M, are reported selectively to inhibit gram-positive micro-organisms (Bergen and Bates, 1984), which include most of the cellulolytic bacteria, capable of hydrolysing fibre (e.g. *Ruminococcus albus*, *Ruminococcus flavefaciens* and *Butyrivibrio fibrisolvens*). In fact, in an *in vitro* degradability experiment, it has been demonstrated that M diminished NDF degradation (Jalc *et al.*, 1992). On the other hand, M also revealed to reduce lactic acid producing bacteria such as *Streptococcus bovis* (McGuffey *et al.*, 2001). In accordance with our results, Martínez *et al.* (2006) reported that two essential oils of thyme at 1.35 µl/ml, one rich in carvacrol and the other in thymol, decreased the *in vitro* potential degradability of NDF (in our study the 250 and 500 mg/l concentrations were equivalent to 0.264 and 0.528 µl/ml of carvacrol).

It must be noted that the R^2 values of the CP models were rather low. This could be due to a bacterial contamination of the bag residue if the washing procedure of the filter bags applied here inappropriate to efficiently remove bacterial residues. As bacteria contain relatively high protein proportions and no NDF, disturbance through bacterial contamination might be of higher importance in CP compared with NDF models.

C500 showed the lowest total VFA concentration. It is likely that the use of such high doses of plant extracts and/or their secondary metabolites with antimicrobial activity decreased total microbial activity and diet fermentability (Cardozo *et al.*, 2004). Moreover, individual VFA proportions were affected by the additives. M changed the VFA profile as expected, increasing propionate proportions and diminishing the ratio acetate:propionate, which agrees with previous findings (Thornton and Owens, 1981). C250 decreased the molar proportion of acetate and propionate, but increased butyrate molar proportion. Evans and Martin (2000) also observed that the use of 400 mg/l of thymol decreased concentrations of CH_4, acetate, propionate and lactate in a mixed ruminal micro-organism fermentation. In addition, Castillejos *et al.* (2006) found that thymol at 5 mg/l tended to reduce the proportion of acetate and increased the proportion of butyrate. These authors also tested thymol at 50 and 500 mg/l, with the former concentration showing no effect on VFA profile, whereas total VFA production has been reduced at 500 mg/l. They concluded that the selection of the optimal dose may require further studies with doses between 5 to 50 mg/l and from 50 to 500 mg/l, to observe positive effects without affecting total VFA concentration.

Shifts in the fermentation pattern provoked through C250 supplementation clearly differed from M supplementation, suggesting that the mechanism of action of C250 is not the same as that of M. In fact, Helander *et al.* (1998) reported that monensin affects mainly some gram-positive bacteria, while essential oils and other vegetal secondary compounds inhibit both gram-positive and gram-negative bacteria.

Although gas production during the present work was not directly evaluated, the molar proportions of acetate, propionate and butyrate were used to predict rumen

https://doi.org/10.1017/S1751731107730781 Published online by Cambridge University Press

methanogenesis (mmol/mol VFA) according to an equation, derived from the stoichiometric relations as described by Demeyer and Fievez (2000). These calculations suggest M supplementation reduced relative methane production, whereas higher methane production was associated with the supplementation of C250 and C500 (291, 241, 325 and 400 mmol CH_4/mol VFA for C, M, C250 and C500, respectively). Therefore, carvacrol at the assayed doses seems to have a different effect compared with M on ruminal methanogenesis.

The action of M on rumen degradability, VFA pattern and rumen methanogenesis was as expected and reported before by other authors. C250 changed the VFA profile, but was different to the fermentation pattern observed with M supplementation. C500 reduced total VFA production. Future research, including *in vivo* studies, in order to understand the factors that contribute to that antimicrobial activity and the selection of the optimal dose is required.

References

Association of Official Analytical Chemists 1990. Official methods of analysis, 15th edition. AOAC, Arlington, VA, USA.

Bergen WG and Bates DB 1984. Ionophores: their effect on production efficiency and mode of action. Journal of Animal Science 58, 1465-1483.

Broudiscou LP, Papon Y and Broudiscou AF 2000. Effects of dry extracts on fermentation and methanogenesis in continous culture of rumen microbes. Animal Feed Science and Technology 87, 263-277.

Busquet M, Calsamiglia S, Ferret A and Kamel C 2006. Plant extracts affect *in vitro* rumen microbial fermentation. Journal of Dairy Science 89, 761-771.

Cancho B, García MS and Simal J 2000. The use of antibiotics in animal feeds: an actual perspective. Ciencia y Tecnología Alimentaria 3, 39-47.

Cardozo PW, Calsamiglia S, Ferret A and Kamel C 2004. Effects of natural plant extracts on protein degradation and fermentation profiles in continuous culture. Journal of Animal Science 82, 3230-3236.

Castillejos L, Calsamiglia S and Ferret A 2006. Effect of essential oil active compounds on rumen microbial fermentation and nutrient flow in *in vitro* systems. Journal of Dairy Science 89, 2649-2658.

Cowan MM 1999. Plant products as antimicrobial agents. Clinical Microbiology Reviews 12, 564-582.

Demeyer D and Fievez V 2000. Ruminants et environnement: la méthanogénèse. Annales de Zootechnie 49, 95-112.

Domescik EJ and Martin SA 1999. Effects of laidlomycin propionate and monensin on the *in vitro* mixed ruminal microorganism fermentation. Journal of Animal Science 77, 2305-2312.

Evans JD and Martin SA 2000. Effects of thymol on ruminal microorganisms. Current Microbiology 41, 336-340.

Helander IM, Alakomi H, Latva-Kala K, Mattila-Sandholm T, Pol I, Smid EJ, Gorris LGM and Wright A 1998. Characterisation of the action of selected essential oil components on Gram-negative bacteria. Journal of Agricultural and Food Chemistry 46, 3590-3595.

Jalc D, Baran M, Vendrak T and Siroka P 1992. Effect of monensin on fermentation of hay and wheat bran investigated by the rumen stimulation technique (Rusitec). Basal parameters of fermentation. Archiv für Tierernährung 42, 147-152.

Juliano C, Mattana A and Usai M 2000. Composition and *in vitro* antimicrobial acitivity of the essential oil of *Thymus herba-barona* Loisel growing wild in Sardinia. Journal of Essential Oil Research 12, 516-522.

Losa R, Frehner M, Newbold CJ and Wallace RJ 2002. Modulation of rumen nitrogen metabolism with essential oil compounds. Proceedings of the fourth Korea-Japan joint symposium on rumen metabolism and physiology. Jeju, Korea, 118.

McGuffey RK, Richardson LF and Wilkinson JD 2001. Ionophores for dairy cattle: current status and future outlook. Journal of Dairy Science 84, (suppl. E) E194-E203.

McIntosh FM, Newbold CJ, Losa R, Williams P and Wallace RJ 2000. Effects of essential oils on rumen fermentation. Reproduction Nutrition Development 40, 221-222, (abstr.).

Madrid J, Megías MD and Hernández F 1999. Determination of short chain volatile fatty acids in silages from artichoke and orange by-products by capillary gas chromatography. Journal of the Science of Food and Agriculture 79, 580-584.

Martínez S, Madrid J, Hernández F, Megías MD, Sotomayor JA and Jordán MJ 2006. Effect of thyme essential oils (*Thymus hyemalis* and *Thymus zygis*) and monensin on *in vitro* ruminal degradation and volatile fatty acid production. Journal of Agricultural and Food Chemistry 54, 6598-6602.

Molero R, Ibars M, Casamiglia S, Ferret A and Losa R 2004. Effects of a specific blend of essential oil compounds on dry matter and crude protein degradability in heifers fed diets with different forage to concentrate ratios. Animal Feed Science and Technology 114, 91-104.

Newbold CJ, McIntosh FM, Williams P, Losa R and Wallace RJ 2004. Effects of a specific blend of essential oil compounds on rumen fermentation. Animal Feed Science and Technology 114, 105-112.

Ørskov ER and McDonald I 1979. The estimation of protein degradability in the rumen from incubation measurements weighted according to rate of passage. Journal of Agricultural Science (Cambridge) 92, 499-503.

Sauvant D, Perez JM and Tran G 2003. Tablas de composición y de valor nutritivo de las materias primas destinadas a los animales de interés ganadero: cerdos, aves, bovinos, ovinos, caprinos, conejos, caballos y peces. 1[a] edición en español. Ediciones Mundi-Prensa, Madrid, Spain.

Sivropoulou A, Papanikolaou E, Nikolaou C, Kokkini S, Lanaras T and Arsenakis M 1996. Antimicrobial and cytotoxic activities of *Origanum* essential oils. Journal of Agricultural and Food Chemistry 44, 1202-1205.

Statistical Packages for the Social Sciences 1997. SPSS 7.5 for Windows. SPSS Inc., Chicago, IL.

Steel GRD, Torrie JH and Dickey D 1996. Principles and procedures of statistics: a biometrical approach. McGraw-Hill, New-York, USA.

Thornton JH and Owens FN 1981. Monensin supplementation and *in vivo* methane production by steers. Journal of Animal Science 52, 628-634.

Van Nevel CJ and Demeyer DI 1977. Effect of monensin on rumen metabolism *in vitro*. Applied and Environmental Microbiology 34, 251-257.

Van Soest PJ, Robertson JB and Lewis BA 1991. Methods for dietary fiber, neutral detergent fiber and nonstarch polysaccharides in relation to animal nutrition. Journal of Dairy Science 74, 3583-3597.

Wang Y, Alexander TN and McAllister TA 2004. *In vitro* effects of Monensin and Tween 80 on ruminal fermentation of barley grain:barley silage-based diets for beef cattle. Animal Feed Science and Technology 116, 197-209.

https://doi.org/10.1017/S1751731107730781 Published online by Cambridge University Press