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## Effects of supplementing a mixed diet with echium (*Echium plantagineum*) oil on methanogenesis in a rumen simulation system

P. Amaro<sup>1</sup>, M.R.G. Maia<sup>1</sup>, R.J. Dewhurst<sup>2</sup>, A.J.M. Fonseca<sup>1</sup> and A.R.J. Cabrita<sup>1,3</sup>

<sup>1</sup> Universidade do Porto, REQUIMTE, ICBAS, Institute of Biomedical Sciences de Abel Salazar  
Jorge Viterbo Ferreira Street 228, 4050-313 Porto, Portugal

<sup>2</sup> Scotland's Rural College, King's Buildings, West Mains Road, Edinburgh EH9 3JG, United Kingdom

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<sup>3</sup> Corresponding author:  
e-mail: arcabrita@icbas.up.pt

**ABSTRACT.** The rumen simulation technique (RUSITEC) was used to evaluate the effect of two levels of echium (*Echium plantagineum*) oil (EO) on methanogenesis. Treatments comprised: control (commercial total mixed ration for lactating dairy cows; positive control), control supplemented with sodium 2-bromoethanesulfonate (5 mM on a dry matter (DM) basis; negative control; BES) and control supplemented with 4% (EO4) and 8% (EO8) of echium oil ( $\text{g} \cdot 100^{-1} \text{g DM}$ ). Methane production and the proportion of methanogens in total rumen prokaryote 16S rDNA from effluents and solid residues were decreased by EO8. Oil supplementation did not affect total volatile fatty acid production and digestibility of DM and neutral detergent fibre, but increased propionate proportion. Despite the *in vitro* results suggesting that echium oil is a promising natural strategy for the mitigation of enteric methane, it is difficult to distinguish whether the observed effects are due to secondary metabolites, a particular fatty acid, or mixtures of fatty acids contained in the echium oil.

### Introduction

Enteric methane is produced by *Archaea* which convert carbon dioxide and hydrogen originating from the rumen fermentation of feedstuffs into methane. Although methanogenesis prevents negative effects of hydrogen accumulation in the rumen, methane emissions have a detrimental effect on the environment and represent a substantial non-productive loss of energy from the animal (e.g., Johnson and Johnson, 1995). Dietary supplementation with lipids is one of several strategies that have been studied to reduce rumen methanogenesis. Supplementation with medium- and long-chain fatty acids

can decrease methane emissions due to a direct inhibition of methanogenic *Archaea* or to an indirect action on the wider microbial community leading to a reduction in fermentable organic matter (Martin et al., 2010). Additionally, vegetable oils rich in polyunsaturated fatty acids (PUFA) can have a toxic effect on the rumen microbial population and provide an alternative electron sink for hydrogen through biohydrogenation (Prins et al., 1972; Czerkawski, 1986).

Echium oil is a vegetable oil extracted from the seeds of *Echium plantagineum*, and is one of the richest sources of stearidonic acid (SDA; C18:4n-3; Guil-Guerrero, 2007). Earlier studies have showed

the potential use of soyabean oil with elevated levels of SDA (Bernal-Santos et al., 2010) and protected echium oil (protein-aldehyde matrix; Kitessa and Young, 2011) to increase the *n*-3 PUFA content in dairy products. Eicosapentaenoic acid (C20:5*n*-3) and docosahexaenoic acid (C22:6*n*-3) are long-chain *n*-3 PUFA known to have beneficial effects on human health (Calder, 2006; Mozaffarian, 2008). These *n*-3 long-chain PUFA are synthesized from  $\alpha$ -linolenic acid (C18:3*n*-3) by a sequence of desaturation and chain elongation steps. Nevertheless, C18:3*n*-3 is poorly converted to C20:5*n*-3 partly because of the low activity of  $\Delta$ 6-desaturase, the rate limiting enzyme for conversion (C18:3*n*-3; Yamazaki et al., 1992). Conversely, as SDA is downstream from  $\Delta$ 6-desaturase, it is more readily converted to C20:5*n*-3 (Kitessa et al., 2014).

Recently, we (Amaro et al., 2012) evaluated the effect of increasing levels of SDA (0, 1, 5, 20 and 50 mg · l<sup>-1</sup> incubation media) on methane production in short-term batch incubations with buffered rumen fluid. Despite the observed shift to increased propionate production at the expense of acetate and butyrate, and the extensive apparent biohydrogenation of SDA, there was no significant reduction in methanogenesis. These results suggest that either higher levels of non-esterified SDA or its supplementation in alternative forms might be needed to achieve methane mitigation. Along with its relatively high SDA content, echium oil is also rich in C18:3*n*-3 and linoleic acid (C18:2*n*-6), known to have potential to decrease methanogenesis (Czerkawski et al., 1966; Zhang et al., 2008). To our knowledge, the potential of echium oil in decreasing methane production has never been reported. Therefore, the present work aimed to evaluate, in an *in vitro* semi-continuous fermentation system (RUSITEC system), the effects of two levels of echium oil supplementation on ruminal methanogenesis and fermentation.

## Material and methods

### Animals

Rumen contents were collected from commercial dairy cows after slaughter at an authorized slaughterhouse (Central Carnes abattoir, Vila Nova de Famalicão, Portugal). Animals were protected at slaughter according to the Council Directive 93/119/EC of 22 December 1993 and rumen content collection for experimental purposes was done by qualified members as previously authorized by the Portuguese National Authority for Animal Nutrition and Health (Direção Geral de Alimentação e Veterinária)

of the Ministry for Agriculture, Sea, Environment and Spatial Planning (Ministério da Agricultura, do Mar, do Ambiente e do Ordenamento do Território, Portugal).

### Semi-continuous *in vitro* incubation

A rumen simulation technique (RUSITEC) system, with eight 800 ml vessels (RUSITEC S, Sanshin Industrial Co. Ltd., Yokohama, Japan), was used as described by Czerkawski and Breckenridge (1977). The inoculum was obtained from three adult dairy cows fed maize silage-based diets immediately after slaughter. Solid and liquid fermentation inocula were placed in a pre-heated thermos container at 39°C for transport to the laboratory and transferred to the RUSITEC system within less than 1 h. At the laboratory, the ruminal digesta was mixed, strained through 4 layers of linen-cloth and maintained at 39°C under O<sub>2</sub>-free CO<sub>2</sub>.

Two identical 12 days incubation trials were carried out independently and the four treatments were assigned randomly within each trial, so that two fermenters received each of the treatments with two replicates per treatment; each treatment was, therefore, performed in quadruplicate. To ensure a steady state within the vessels, each of the two incubation trials consisted of 4 days for stabilization followed by 8 days of data collection. A period serving for the adaptation of the rumen microbes to the fermentation system and diet has been previously found to be the best compromise due to the constraints to extend the experimental periods over more days (Machmüller et al., 1998).

On the first day of each period, vessels were incubated with 500 ml strained rumen fluid, 300 ml artificial saliva (McDougall, 1948), 70 g strained rumen solids and 12 g basal diet (dry matter, DM, basis) placed in individual nylon bags (80 × 120 mm, 50 µm pore size; Sanshin Industrial Co. Ltd., Yokohama, Japan). The basal diet was a commercial total mixed ration (TMR) for lactating dairy cows, with 491 g maize silage, 85 g wheat straw and 424 g concentrate mixture (per kg, DM basis; Table 1), dried at 65°C for 48 h and milled through a 4 mm sieve.

After 24 h of incubation, the bag containing rumen solids was replaced by a bag containing the basal diet. Each vessel contained two feed bags that remained in the system for 48 h, with alternate bags replaced every 24 h. Treatments were introduced on day 5 and were fully implemented from day 6 onwards. The four treatments were each allocated to two fermenter vessels: control (basal diet; positive control), control supplemented with sodium 2-bromoethanesulfonate (5 mM on a DM

**Table 1.** Composition of the total mixed ration (TMR) used as substrate

Item	TMR
Ingredient, g · kg <sup>-1</sup> DM	
maize silage	491
wheat straw	84.7
Concentrate mixture	
maize grain	123
barley	8.85
wheat bran	19.8
soyabean meal	157
rapeseed meal	50.9
sunflower meal	7.78
expeller palm kernel meal	7.43
calcium soaps	11.44
molasses	9.19
urea	2.54
slow release urea <sup>1</sup>	1.69
calcium carbonate	9.18
dicalcium phosphate	2.97
salt	2.12
sodium bicarbonate	5.51
magnesium oxide	2.12
mineral–vitamin premix <sup>2</sup>	2.54

<sup>1</sup> Optigen, Alltech, Sintra, Portugal; <sup>2</sup> contained, per kg, IU: vit. A<sub>2</sub> 500.000, vit. D<sub>3</sub> 416.666; mg: vit. E 12.500, vit. B<sub>3</sub> 66, vit. B<sub>5</sub> 100.000, vit. H 333, beta-carotene 333, Co 166, Cu 1.666, Fe 5.000, I 333, Mg 83.333, Mn 10.000, Se 50, Zn 16.666, *Saccaromyces cerevisiae* (Yea-Sacc 1026, Alltech, Sintra, Portugal) 300,000,000 CFU, and mycotoxin adsorbent (Mycosorb, Alltech, Sintra, Portugal) 500

basis; negative control; BES), and control supplemented with 4% (EO4) and 8% (EO8) of echium oil (w/w). Sodium 2-bromoethanesulfonate was used as a negative control as it is a halogenated analogue of coenzyme-M that inhibits methanogenesis by suppressing the methyl-coenzyme M reductase in *Archaea* (Balch and Wolfe, 1979; Zhang and Yang, 2011). The supplements were added daily to the basal diet just prior to the introduction of feed bags into the RUSITEC vessels.

The system was flushed with O<sub>2</sub>-free CO<sub>2</sub> during the daily exchange of feed bags. Artificial saliva was continuously infused at a rate of 0.045 l · h<sup>-1</sup> and gas and liquid effluents collected daily in gas-tight collection bags (Tedlar bag, Tokyo Deodorant Co., Tokyo, Japan) and in flasks placed in an ice-cold water-bath, respectively. Daily total gas production from each fermenter was measured with a dry gas meter (model DC-1A, Shinagawa Corporation, Tokyo, Japan) and 5 ml samples collected for determination of gas composition. The pH (electrode 5029) and redox potential (electrode ORP 5058) of fermentation media were measured when vessels were opened for exchange of feed bags. Measurements of

pH were also made in effluents (GLP22+ pH meter, Crison Instruments, Spain). Liquid effluent was collected and ammonia-N (NH<sub>3</sub>-N) and volatile fatty acids (VFA) concentrations were determined. For VFA analysis, 1 ml effluent was added to 0.25 ml 25% ortho-phosphoric acid solution with internal standard (16 mM 3-methyl valeric acid; Sigma-Aldrich Inc., St. Louis, MO, USA) and kept at -20°C. For determination of NH<sub>3</sub>-N concentration, 10 ml of effluent was added to 10 ml of 0.1 M HCl solution and samples were kept at 4°C until analysis.

The feed bag residues were collected during the adaptation and the experimental days and kept at -20°C until further analysis. Apparent DM and neutral detergent fibre (assayed with a heat stable amylase and expressed exclusive of residual ash; NDF) digestibilities determined in whole residue were performed on days 3, 4, 8 and 9; and the proportion of methanogens in the total prokaryote population was determined on days 5, 10, 11 and 12.

### Chemical analysis

The chemical composition (DM, ash, NDF, Kjeldahl N, ether extract, starch and urea) of the basal diet and fatty acids in the basal diet and echium oil were performed as described by Amaro et al. (2012).

Feed bags were rinsed with cold water in a washing machine, dried at 65°C for 48 h, and weighed to determine DM apparent digestibility and further NDF apparent digestibility (method 973.18; AOAC, 1990). Effluent NH<sub>3</sub>-N concentration was determined by titration after Kjeldahl steam distillation (method 954.01; AOAC, 1990) and VFA concentrations, gas composition and methane concentration analysed by gas chromatography (Amaro et al., 2012).

### DNA extraction and Real-Time PCR analysis

Feed residues and effluent samples were freeze-dried and composited for each fermenter vessel across experimental days and across vessels for the adaptation period. Total genomic DNA was extracted after homogenization and physical disruption using a bead beater (Mini-bead Beater, BioSpec Products, Bartlesville, OK, USA), following the method described by Yu and Morrison (2004) with the modification of a higher temperature (85°C) at the cell lysis step. The concentration of DNA in the extract was measured by spectrophotometry (Nanodrop ND-1000, Nanodrop Technologies, Wilmington, DE, USA).

Quantitative Real-Time PCR (qPCR) was performed on a 7500 Fast Real-Time PCR System (Applied Biosystems, Dublin, Ireland), with fluo-

rescence detection of SYBR Green dye. The primer sets used in the present study to amplify partial 16S rRNA gene regions of the microbial genome were selected on the basis of a thorough review of the published literature (e.g., Edwards et al., 2005). The chosen V3 and the methanogen *mcrA* primers were described by Muyzer et al. (1993) and Denman et al. (2007), respectively. Despite being designed for denaturing gradient gel electrophoresis, V3 primers have been successfully used in qPCR (Brankatschk et al., 2012; Carberry et al., 2012). Extracted DNA was diluted to a concentration of  $1 \text{ ng} \cdot \text{l}^{-1}$  and used as a template in all PCRs. Polymerase chain reactions were done in triplicate, using 20  $\mu\text{l}$  reaction mixtures of 1  $\mu\text{l}$  DNA, 10  $\mu\text{l}$  Fast SYBR® Green Master Mix (Applied Biosystems, Dublin, Ireland), 1  $\mu\text{l}$  forward and reverse primers (10  $\mu\text{M}$ ) and 8  $\mu\text{l}$  nuclease-free water. The programme used for qPCR was as follows: 1 cycle at 95°C for 20 s for enzyme activation, 40 cycles at 95°C for 3 s and 60°C for 30 s, followed by 1 cycle at 95°C for 15 s 60°C for 1 min, 95°C for 15 s, and 65°C for 15 s. Negative controls without template DNA were included in parallel.

Due to the inconsistency in the concentration of PCR inhibitors in each sample, the efficiency of the reaction to ensure correct quantitation was estimated. From an initial concentration of  $10 \text{ ng} \cdot \mu\text{l}^{-1}$  of DNA, five serial dilutions (10-fold) were performed. Amplification efficiencies (E) were estimated from a linear regression of the threshold cycle (CT) for each dilution versus the log dilution using the formula:  $E = [10^{-(1/\text{slope})} - 1] \times 100$ . Efficiency for the V3 primer ranged from 94% to 101% and 86% to 94%, respectively, for effluents and residues. Efficiency for *mcrA* primers varied between 93% to 101% and 93% to 99%, respectively, for effluents and residues. The slope varied between -3.3 and -3.5 for V3 and *mcrA* in the effluent samples and between -3.5 and -3.8, and -3.3 and -3.5, respectively, for V3 and *mcrA* in residues.  $R^2$  was 0.99 in effluents for both primers and ranged between 0.98 and 1, and 0.99 and 1 in residues, respectively, for V3 and *mcrA*. Methanogen population was estimated as a proportion of total rumen prokaryote 16S rDNA, according to Zhang et al. (2008):  $\Delta Ct = (2 - (Ct \text{ methanogenes} - Ct \text{ total bacteria}) \times 10^3)$ .

### Statistical analysis

All data were analysed using the MIXED procedure of the SAS (2002) software (version 9.1, SAS Institute Inc., Carry, NC). The statistical model included the fixed effect of treatment (control, BES, EO4 and EO8), and the random effect of period.

Adaptation days measurements were used as covariates, the covariance structure being chosen according to Akaike and Schwarz's Bayesian information criteria (Wang and Goonewardene, 2004). Covariates were removed from the model when their effect was not significant ( $P > 0.15$ ). Orthogonal contrasts were constructed for all data in order to compare effects between treatments. Effects were considered significant when  $P \leq 0.05$  and a trend when  $0.05 < P \leq 0.10$ .

## Results

The TMR used in the experiment contained (on a DM basis)  $179 \text{ g} \cdot \text{kg}^{-1}$  crude protein (Kjeldahl  $N \times 6.25$ ; CP),  $383 \text{ g} \cdot \text{kg}^{-1}$  NDF and  $252 \text{ g} \cdot \text{kg}^{-1}$  starch, the major fatty acids found being C16:0, *cis*-9 C18:1 and C18:2*n*-6 (Table 2). Echium oil contained 15% SDA, other fatty acids present in significant amounts being *cis*-9 C18:1, C18:2*n*-6, C18:3*n*-6 and C18:3*n*-3 (Table 2).

The addition of 8% echium oil (EO8) and BES decreased total gas production, as well as production of methane. Methane was only detected from day 3 of the experimental period in the fermenters with BES (data not shown). The proportion of methanogens

**Table 2.** Chemical composition and fatty acid profile of the total mixed ration (TMR) and echium oil

Item	TMR	Echium oil
Dry matter (DM), $\text{g} \cdot \text{kg}^{-1}$	970	
Chemical composition, $\text{g} \cdot \text{kg}^{-1}$ DM		
ash	65.0	
crue protein	179	
ether extract	30.0	
NDF	383	
starch	252	
urea	5.40	
Fatty acids, $\text{g} \cdot 100 \text{ g}^{-1}$ total fatty acids		
C12:0	1.32	–
C14:0	1.12	–
C16:0	22.2	6.88
<i>cis</i> -9 C16:1	0.418	0.080
C18:0	2.33	3.14
<i>cis</i> -9 C18:1	23.5	14.4
<i>cis</i> -11 C18:1	1.83	0.640
C18:2 <i>n</i> -6	39.7	13.3
C18:3 <i>n</i> -6	–	10.8
C20:0	0.329	–
C18:3 <i>n</i> -3	6.29	34.8
C18:4 <i>n</i> -3	0.409	15.0
other fatty acids <sup>1</sup>	0.531	1.18

<sup>1</sup> the remaining unidentified fatty acids

**Table 3.** Mean effects of basal diet (control), control supplemented with sodium 2-bromoethanesulfonate (5 mM DM basis; negative control; BES), and control supplemented with 4% (EO4) and 8% (EO8) of echium oil ( $\text{g} \cdot 100 \text{g}^{-1}$  DM) incubated in a long-term semi-continuous fermentation system with buffered rumen fluid (RUSITEC) on gaseous emissions, proportion of methanogens, pH, ammonia-N ( $\text{NH}_3\text{-N}$ ) concentration, and volatile fatty acid (VFA) concentration and proportions

Indices	Treatment				SEM	Contrast					
	Control	BES	EO4	EO8		CvsBES	BESvsEO4	BESvsEO8	CvsEO4	CvsEO8	EO4vsEO8
<b>Gaseous emissions</b>											
total gas, ml	2108	1773	2302	1680	272.1	0.038	0.003	0.524	0.201	0.012	0.001
methane, ml	138	8	134	57	34.7	0.001	0.001	0.097	0.906	0.014	0.018
methane · total gas <sup>-1</sup> , %	6.25	0.47	5.48	3.21	0.984	<0.001	<0.001	<0.001	0.107	<0.001	<0.001
<b>Methanogens<sup>1</sup></b>											
effluents	1.96	0.33	2.27	0.69	0.450	<0.001	<0.001	0.337	0.409	0.004	0.001
residues	1.38	0.29	1.01	0.75	0.222	0.002	0.024	0.124	0.212	0.043	0.359
<b>Fermenter vessels</b>											
pH	6.67	6.69	6.68	6.71	0.112	0.633	0.876	0.557	0.747	0.296	0.460
redox, mV	-322	-311	-323	-322	2.0	0.002	0.001	0.003	0.870	0.760	0.641
<b>Effluents</b>											
pH	6.71	6.69	6.66	6.66	0.073	0.566	0.384	0.236	0.139	0.097	0.801
$\text{NH}_3\text{-N}$ , $\text{mg} \cdot \text{ml}^{-1}$	135	111	148	130	18.4	0.034	0.003	0.082	0.224	0.621	0.100
Total VFA, $\text{mmol} \cdot \text{l}^{-1}$	65.9	62.5	68.2	69.2	11.11	0.483	0.249	0.181	0.640	0.504	0.829
<b>Molar proportion, <math>\text{mol} \cdot 100 \text{mol}^{-1}</math></b>											
acetate	48.9	47.6	48.9	47.3	2.04	0.523	0.485	0.857	0.970	0.449	0.401
propionate	20.9	20.6	22.5	24.3	2.66	0.823	0.097	0.004	0.140	0.006	0.110
isobutyrate	1.05	0.82	1.00	0.90	0.051	0.003	0.014	0.202	0.388	0.031	0.143
butyrate	17.9	19.1	17.5	15.8	3.26	0.369	0.251	0.023	0.788	0.116	0.181
isovalerate	3.86	4.74	4.01	4.51	0.141	0.001	0.003	0.274	0.495	0.008	0.027
valerate	4.95	5.45	5.23	5.85	0.598	0.527	0.779	0.615	0.723	0.282	0.465
isocaproate	0.04	0.06	0.04	0.04	0.022	0.084	0.133	0.055	0.711	0.804	0.541
caproate	1.49	1.32	1.25	1.22	0.369	0.407	0.741	0.635	0.254	0.204	0.885
acetate:propionate	2.40	2.34	2.26	1.96	0.152	0.732	0.656	0.043	0.435	0.023	0.094
<b>Digestibility, %</b>											
dry matter	75.9	74.9	74.7	75.3	2.19	0.710	0.951	0.869	0.665	0.835	0.821
NDF	47.8	42.6	45.8	44.4	4.49	0.436	0.626	0.783	0.755	0.605	0.829

<sup>1</sup> methanogens estimated as a % of total bacterial 16S rDNA;  $\Delta\text{Ct} = (2 - (\text{Ct}_{\text{methanogenes}} - \text{Ct}_{\text{total bacteria}}) \times 10^3)$

within the prokaryote DNA isolated from effluents and residues was lower for the EO8 and BES treatments, the effect of EO8 also being similar to EO4 for residues (Table 3). Treatments had no effect on the pH of fermenter vessels or effluents, or on DM and NDF digestibilities, but BES treatment increased redox potential and decreased  $\text{NH}_3\text{-N}$  production (Table 3). Total VFA concentration was not affected by treatments. Propionate proportion was higher for the EO8 treatment compared to the control and BES treatment, no differences being observed for EO4. The acetate:propionate ratio decreased with EO8 relative to control and BES, though it was not different from EO4.

## Discussion

*Echium plantagineum* is mainly known as a weed of pastures found in regions of the world with a Mediterranean-type climate. It is grown commercially in Europe as a winter annual plant (occasionally biennial) and is readily eaten by livestock in the early stages of growth (Cabi, 2014). The key problem associated with *E. plantagineum*, is that it contains pyrrolizidine alkaloids that can cause hepatotoxic photosensitization and animal mortality, monogastric animals being more susceptible to alkaloid poisoning. *Echium plantagineum* is currently one of the best agricultural source of SDA and C18:3n-3, quite valuable

as health supplements to humans, constituting a potential alternative to marine oils as dietary sources of *n*-3 PUFA (Surette et al., 2004). Echium oil has many potential uses in the pharmaceutical industry for treatment of eczema, acne and other skin disorders, and it is also valued for its moisturizing and anti-inflammatory action (McEwen et al., 2003), being highly available on the market. The current market price of echium oil is unknown since it is used as a source of SDA and C18:3*n*-3, but has been closely linked to the prices of the most common sources of C18:3*n*-3, including evening primrose (*Oenothera biennis*) and borage (*Borago officinalis*) oils (Berti et al., 2007).

In the present study, the effects of echium oil supplementation on methanogenesis were evaluated in RUSITEC, in comparison to those of positive (basal diet) and negative (BES) controls. Methane production was only detected on day 3 of the experimental period for the BES treatment (data not shown), as earlier observed by Karnati et al. (2009) with 250  $\mu$ M of BES, and suggesting an adaptation of the methanogenic population to the halogenated compound. Sodium 2-bromoethanesulfonate reduced methane production by 92.8%, compared to the control, a similar finding (95%) being described for the same supplementation level *in vitro* (Lee et al., 2009).

The depression in methane production by PUFA *in vitro* and *in vivo* is well established. In the present study, supplementation with 8% echium oil reduced daily methane production by 60%, but the lower level (4%) was unable to reduce methanogenesis. It is difficult to distinguish whether the effects of oil supplementation in methane mitigation are due to secondary metabolites, a particular fatty acid, or mixtures of fatty acids. Secondary compounds, like the pyrrolizidine alkaloids echimidine and echiumine, known to contribute to liver damage and photosensitivity in grazing livestock (Weston et al., 2013), are found in greatest quantity in *E. plantagineum* (Colegate et al., 2005). There are marked differences in the sensitivity of domestic species to echium toxicosis, with cattle and horses showing the highest levels of sensitivity (Cheeke, 1984). Although the rumen microorganisms that may be able to detoxify these alkaloids have not yet been identified, Wachenheim et al. (1992) found that jacobine biotransformation involves multiple bacterial species and Gram-positive bacteria can play a key role. However, as pyrrolizidine alkaloids are not lipophilic, they are not expected to be present in substantial quantities in the oil.

Echium oil contains relatively high levels of C18:3*n*-3, C18:4*n*-3, *cis*-9 C18:1 and C18:2*n*-6. Linoleic and  $\alpha$ -linolenic acids are known to reduce methane production, the latter having a more pronounced effect (Dohme et al., 2001). Recently, Amaro et al. (2012), using SDA inclusion levels up to 6.25 g  $\cdot$  kg<sup>-1</sup> TMR, failed to detect significant effects on methane production (mmol g  $\cdot$  kg<sup>-1</sup> DM incubated), the higher level of supplementation corresponding to the SDA present in 4–5% of echium oil addition, toxic effects only being reported at supplementation levels of 30 g SDA  $\cdot$  kg<sup>-1</sup> substrate DM and greater (Maia et al., 2012). Therefore, the present results obtained with 8% echium oil addition might be explained by a higher supply of PUFA, known to exert toxic effects on the rumen microbial population (Prins et al., 1972; Maia et al., 2007), greater effects being reported with increased unsaturation (Demeyer and Henderickx, 1967). Indeed, EO8 decreased the proportion of methanogens relative to rumen prokaryote 16S rDNA from effluents, the effect being similar to that for BES. The effect of lipid supplementation level (EO4 vs EO8) on the methanogen population was not observed in the solid samples (feed bag residues). Several studies (Hook et al., 2011; Lillis et al., 2011) have reported higher numbers of methanogens in rumen solids than in rumen liquid regardless of diet. However, diet may affect the distribution of methanogens between the solid- and liquid-phases of the rumen, with important implications for methanogenesis (McCartney et al., 2013). In the present study, despite not being significant, when compared to control, EO4 increased the proportion of methanogens in the effluents and decreased the proportion of methanogens in residues, suggesting a change in the microbial communities between liquid and solid phases. Indeed, diet has been suggested to mainly affect bacterial community abundance and diversity, whereas only the abundance, and not the diversity, of methanogens is affected (Lillis et al., 2011). Changes in bacterial populations might result in different fermentation patterns and therefore decreased methane production as a consequence of reduced substrate supply (hydrogen; Stewart et al., 1997). Despite the absence of effects of oil inclusion on total VFA production and DM and NDF digestibilities, echium oil increased the production of propionate (which is the main alternative sink for metabolic hydrogen during rumen fermentation), lowered the acetate:propionate ratio and decreased butyrate.

A similar fermentation shift towards propionate production with lipid supplementation was reported in earlier studies (Wettstein et al., 2000).

## Conclusions

The *in vitro* results suggest that echium oil supplementation is a promising natural strategy for enteric methane mitigation. However, the content of secondary metabolites, stearidonic and a mixture of PUFAs of echium oil may all have contributed to the observed effects of oil supplementation in methane mitigation.

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