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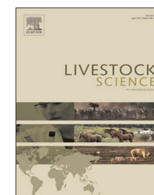

In vivo and *in vitro* effects of a blend of essential oils on rumen methane mitigation

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ABSTRACT

The effect of Agolin Ruminant, a blend of essential oils, on methane (CH_4) emissions were investigated in two *in vivo* experiments and in four *in vitro* experiments. In the *in vivo* experiments, four lactating dairy cows and four beef heifers were supplemented 0.2 g/d of the essential oils (*ca.* 2–4 ppm m/v) during an eight-weeks period, where the first two weeks served as control (no essential oils supplementation). In dairy cattle, essential oils tended to decrease the daily CH_4 emissions (g/d) and CH_4 relative to dry matter intake (g/kg DMI) by 15% and 14%, respectively, after 6 weeks of supplementation ($P=0.07$), but no difference was observed for CH_4 relative to milk production (g/kg milk) ($P=0.64$) or CH_4 relative to bodyweight (g/kg BW) ($P=0.12$). In the *in vivo* experiment with beef cattle daily CH_4 emissions and CH_4 relative to DMI did not change when supplemented the essential oils at a dose of 0.2 g/d (numerical decreases of 10 and 11% for g CH_4/d and g CH_4/kg DMI, respectively) but CH_4 relative to body weight tended to decrease by 20% after 6 weeks of supplementation ($P=0.07$). The *in vitro* experiments were expected to replicate the results observed *in vivo*. However, no decrease in CH_4 production was observed in 24 h batch incubations at concentrations up to 30 ppm (m/v). A longer contact time between the essential oils (15 and 30 ppm) and the feedstuff (essential oils added *ca.* 16 h prior the start of the incubation) did not elicit any effect on CH_4 production and was not different from addition immediately prior to the start of the incubation. Longer incubation time (96 h and 14 d) and regular supply of both substrate and additive in a consecutive batch incubation system did not induce CH_4 inhibition up to essential oils doses of 30 ppm (m/v) and hence, also were not able to replicate *in vivo* results. Using the gas production technique (GPT) methane was inhibited by 17% with an essential oils dose of 30 ppm after 24 h, but this decrease was not constant across all times during the 72 h incubation. The blend of essential oil was effective reducing daily emissions of methane in dairy cattle and emissions relative to body weight in beef cattle, interestingly, these effects were not observed *in vitro* regardless of the techniques used to replicate *in vivo* results. This might be due to differences in the mode of action of the essential oils *in vitro* and *in vivo*, which merits attention for future research.

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1. Introduction

In 2012, the agricultural sector worldwide emitted around 5.4 billion tons (CO_2 eq.) greenhouse gases, with enteric fermentation representing around 39% of those emissions (FAOSTAT, 2014). Methane emissions from agricultural activities represented 77% of the total Belgian methane emissions in 2010 (UNFCCC, 2011a), with 68% of agricultural methane originating from enteric fermentation (UNFCCC, 2011b). Therefore, it exists a global interest to decrease methane (CH_4) emissions from ruminants. In this regard, antimicrobial compounds like, ionophores are known to have anti-methanogenic effects in dairy and beef cattle, with variable results in the magnitude of the inhibition and the persistence of their inhibitory effects (Beauchemin et al., 2008). However, the prohibition to use antibiotics in animal feeding in Europe has

Abbreviations: BW, body weight; DMI, dry matter intake; GPT, gas production technique; VFA, volatile fatty acids

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prompted the search for alternatives to these products and the research on plant derived compounds to modulate ruminal fermentation. In general, a large number of plant compounds are screened *in vitro*, and the results from promising compounds are later validated *in vivo* (Beauchemin et al., 2008).

Essential oils are plant secondary metabolites believed to have potential as dietary additives, due to their strong antibacterial properties (Burt, 2004). Essential oils inhibited the energy metabolism of *Streptococcus bovis* and *Selenomonas ruminantium* (Evans and Martin, 2000) and the growth of *Methanobrevibacter smithii*, a rumen Archaea (McIntosh et al., 2003). The latter is of major interest given worldwide attempts to decrease CH_4 from ruminants.

Essential oils have been studied both *in vivo* and *in vitro*, but effects on CH_4 were variable, which may be linked to the large diversity in the nature of these compounds (Calsamiglia et al., 2007). Hence, routine screening is required to assess the effectiveness of numerous essential oils and blends at different doses. *In vitro* batch incubation systems are practical tools for this purpose. However, in some cases *in vitro* observations do not reflect the results found *in vivo* (Flachowsky and Lebzien, 2012), making it challenging to transform effective *in vitro* concentrations to *in vivo* doses. Given the current interest on rumen CH_4 production, the focus of this study is to test the effects of a blend of essential oils on CH_4 production *in vivo* with both beef and dairy cattle, and *in vitro* using different techniques.

2. Materials and methods

Procedures with animals were approved by the ethical commission of the Institute for Agricultural and Fisheries Research (ILVO), Belgium (Reference number EC 2011-154).

2.1. Materials

Additive. The commercial blend of essential oils provided by AGOLIN SA (Bière, Switzerland) contained 200 g/kg (m/m) of active compounds, mainly coriander oil, geranyl acetate and eugenol. From now onwards when referring to doses and concentrations, we will mean the amount of active compounds supplemented (*e.g.* 0.2 g of essential oils, when the amount of the commercial blend of essential oils added was 1 g).

2.2. In vivo experiments

2.2.1. Blend of essential oils experiment with dairy cattle

Four multiparous lactating Holstein dairy cows with an average body weight of 603 kg (±70.0) and being 296 (±97.6) days in milk at the start of the experiment were used for the *in vivo* measurements. The experiment ran over a period of eight-weeks and was performed between October and December 2011.

Before the start of the experiment, cows had *ad libitum* access to a mixture of grass silage (460 g/kg DM), maize silage (370 g/kg DM) and soybean meal (50 g/kg DM) and were supplemented with concentrate (120 g/kg DM) (Table 1). The proportions of the offered feedstuffs during this period were equal to those during the experiment. Before the start of the experiment a fixed amount (95% of the *ad libitum* intake of the cow with the lowest intake) was offered daily until the end of the measurements. This was done to avoid confounding effects (*e.g.* treatment and advanced lactation stage) on feed intake and passage rate in the rumen. The proportions of feedstuffs along with their chemical composition are presented in Table 1. The first two weeks of the experiment the animals were fed the basal diet (Control period); the blend of essential oils was fed starting on the third week of the experiment. Cows were fed and milked twice a day at 0730 AM and 0530 PM and had free access to drinking water at every time throughout the experiment.

2.2.2. Blend of essential oils experiment with beef cattle

Four Belgian Blue double muscled beef heifers with an average body weight of 484 kg (±111.3 kg) at the start of the experiment were used for the *in vivo* measurements. The eight-week experiment was performed between October and December 2012.

Two weeks before the experiment, the animals were fed maize silage *ad libitum* and supplemented with concentrate. The proportions (Table 1) during this period were equal to those during the experiment. Similar to the trial with dairy cattle, before the start of the experiment, a fixed amount (95% of the *ad libitum* intake of the heifer with the lowest intake) was offered daily until the end of the experiment. The first two weeks of the experiment the animals were fed the basal diet (Control period); the blend of essential oils was fed starting on the third week of the experiment. Heifers were fed once daily at 0730AM and had free access to drinking water throughout the experiment.

2.2.3. Blend of essential oils supplementation

For both beef and dairy cattle the blend of essential oils was supplemented daily starting after the gas measurements of the

Table 1
Ingredient and chemical composition of the experimental diets (g/kg DM) as offered to both dairy cows and beef heifers.

	Dairy cattle				Beef cattle	
	Grass silage	Maize silage	Concentrate	Soybean meal	Maize silage	Concentrate
Dietary proportion (g/kg DM)	460	370	120	50	500	500
Chemical composition (g/kg DM)						
Dry matter (g/kg fresh matter)	313	376	877	862	373	876
Crude protein	171	83	189	516	71	212
Crude fat	43	29	31	33	38	79
Crude ash	100	41	84	64	65	76
Sugars	25	0.4	105	109	n.d.	83
Starch	n.d.	418	219	n.d.	382	34
Neutral detergent fiber	319	458	208	158	346	268
Acid detergent fiber	178	286	135	122	195	155
Acid detergent lignin	13.3	16.6	37.5	37.0	15.8	21.6
Organic matter	900	959	916	936	965	924
Organic matter digestibility (g/kg DM)[a]	799	787	898	907	753	812

[a] *In vivo* organic matter digestibility estimated from cellulose digestibility.

control period (week 0). Each animal received a daily dose of 200 mg essential oils, which was homogenously mixed with part of the concentrate.

2.2.4. Methane measurements

Enteric CH_4 was measured in weeks 0, 2, 4 and 6 by keeping the animals in individual open circuit chambers (De Campeneere and Peiren, 2014). Measurements in week 0 (no blend of essential oils) were considered as control for this experiment. Each measuring period lasted from Tuesday morning until Friday morning.

Through ventilation, a slight negative atmospheric pressure was generated inside the chamber to avoid leakage of air from the chamber. Each chamber has a volume of 12.3 m^3, this volume was completely exchanged by the ventilation system (400 m^3/h) approximately every 2 min. Samples of air were taken from the outlet of the chamber through a tube connected to an eight-channel multi-sampler directly attached to the gas analyzer (infrared laser optical-feedback cavity-enhanced absorption spectrometer). Methane was determined every second. Each channel was monitored during 180 s. The average of the last 60 s was used as a data point. Background air outside the chambers was also sampled and analyzed by two additional channels.

2.3. In vitro experiments

2.3.1. Substrates

Three batches of the mixtures of concentrate+grass silage+maize silage and of the mixture maize silage+concentrate were used as substrates for the *in vitro* experiments. The chemical composition of these mixtures is presented in Table 2.

2.3.2. Experiments

Simultaneous to the *in vivo* trials, three different techniques were used to study the effects of the blend of essential oils on *in vitro* CH_4 formation. Experiment 1 was performed using two different feed mixes as substrates: concentrate+maize silage (50:50, DM basis) and concentrate+grass silage+maize silage (30:35:35, DM basis), whereas in all of the other *in vitro* experiments only the latter substrate was used.

Experiment 1. . *Standard 24 h batch incubations with increasing essential oils concentrations.*

The blend of essential oils was incubated during 24 h (Castro-Montoya et al., 2012) delivering concentrations of 0, 0.25, 0.5, 1, 2, 5, 15 and 30 ppm (volume basis) in triplicate.

Experiment 2. . *Standard 24 h incubation with varying moment of essential oils addition.*

In this experiment, 24 h batch incubations were performed using essential oils at levels of 15 and 30 ppm and testing the effect of the moment of addition of the aqueous solution with essential oils. In the standard set-up the additive is added to the *in vitro* batch system shortly before the start of the incubation. This was compared with the addition of the additive the afternoon before the start of the experiment (*ca*. 16 h earlier).

Table 2
Chemical composition of the substrates used for *in vitro* experiments (g/kg DM). Three different mixtures were used for each type of substrate.

	Concentrate: Grass Silage: Maize Silage			Concentrate: Maize Silage		
	Mix 1	Mix 2	Mix 3	Mix 1	Mix 2	Mix 3
Crude protein	151	163	155	130	148	136
Crude fat	57.9	52.6	41.1	56.5	51.8	41.6
Crude ash	86.4	85.4	73.1	65.7	56.2	58.5
Neutral detergent fiber	490	486	492	448	397	369
Acid detergent fiber	258	270	224	225	215	166
Acid detergent lignin	41.5	51.7	34.3	42.9	40.3	24.6

Experiment 3. . *Consecutive batch incubation*

In a first series within the consecutive batch incubations (Theodorou et al., 1984) the blend of essential oils was tested at two concentrations (15 and 30 ppm) with transfer every 24 h for a total incubation time of 96 h. In a second series of consecutive batch incubations, the incubation time was extended to 14 days with transfers every 48 h. The blend of essential oils was tested at three concentrations (5, 15 and 30 ppm).

Experiment 4. . *Gas production technique, 72 h.*

The blend of essential oils was incubated at concentrations of 15 and 30 ppm during 72 h with CH_4 measurements after 2, 4, 8, 12, 24, 30, 36, 48, 58 and 72 h using the gas production technique (GPT) (Cone et al., 1996).

For experiment 1, 2 and 4 incubations were done in triplicates within one run. The first series of experiment 3 included 6 replicates divided in two runs in different weeks. The second series of experiment 3 included 5 replicates within the same run.

Methane formation was measured in all experiments, whereas total VFA was measured in experiment 1, experiments 2 and 4, and in the second series of experiment 3.

2.3.3. In vitro methods

2.3.3.1. Standard in vitro 24 h batch incubation. The rumen fluid was collected before the morning feeding from three rumen fistulated sheep. The sheep were fed hay *ad libitum* and had free access to drinking water. Fistulation of the sheep had been approved by the ethical commission of the Institute for Agricultural and Fisheries Research (ILVO), Belgium (Reference number EC 2009–114). The rumen fluid obtained from the sheep was brought in insulated flasks for transport and was mixed, homogenized and filtered through a sieve with a pore size of 1 mm under continuous CO_2 flushing and kept in a water bath at 39 °C to be used as the source of inoculum.

The *in vitro* batch incubation method was as described by Fievez et al. (2005). Briefly, 250 mg of the dried corresponding substrate was incubated in 120 ml capacity gastight incubation flasks flushed with CO_2 having 20 ml of a phosphate buffer (per liter of distilled water: 28.8 g Na_2HPO_4 $12H_2O$; 6.1 g NaH_2PO_4 H_2O; 1.4 g NH_4Cl, flushed with CO_2 for 1 h and adjusted to pH 6.8) and 5 ml rumen fluid in a batch culture incubator (Edmund Bühler Gmbh, Hechingen, Germany). Before adding the buffer solution, a freshly prepared aqueous solution of Agolin Ruminant was added to each flask to reach the desired concentration in 25 ml of the medium. Fermentation flasks without additives, but containing 250 mg of the corresponding substrate, were used as a control. After 24 h at 39 °C, flasks were removed from the incubator, placed in ice water to stop the fermentation and sampled for gas, pH and VFA determination.

2.3.3.2. Consecutive batch incubation. The effect of essential oils on CH_4 formation was tested using a modification of the consecutive batch culture technique first described by Theodorou et al. (1984). The first incubation run was prepared as described in Section 2.3 and incubated for 24 h. The following incubation flasks contained 250 mg of substrate, essential oils and 20 ml of phosphate buffer plus clarified rumen fluid (3:1, v/v). Each incubation flask received 5 ml inoculum from the previous incubation. For the first series of

experiment 3, three transfers were done for a total of 96 h incubation. In the second series of experiment 3, this methodology was adapted to allow for transfers of inoculum every 48 h for a total of 14 days incubation time.

2.3.3.3. Gas production technique (GPT). Gas production incubations were performed in the facilities of the Animal Nutrition Group, Department of Animal Sciences of Wageningen University (The Netherlands) following the procedure of Cone et al. (1996). Rumen fluid was collected from two lactating rumen cannulated Holstein Friesian cows, 2 h after the morning feeding. Rumen fluid from both cows was combined and stored in warm insulated flasks filled with CO_2, filtered through two layers of cheesecloth, and mixed (1:2, v/v) with an anaerobic buffer/mineral solution as described by Cone et al. (1996). The blend of essential oils was incubated with 0.5 g DM of substrate in triplicate in 60 ml buffered rumen fluid in 250 ml bottles modified for gas sampling as described by Pellikaan et al. (2011) in a shaking water bath at 39 °C and gas production was recorded for 72 h. Gas production measurements were corrected for blank gas productions (*i.e.*, gas productions in buffered rumen fluid without sample). At the end of the incubation time the flasks were opened and 2 ml of the incubation medium was sampled, acidified and prepared for VFA analysis.

2.3.3.4. Analysis of metabolites during in vitro incubations

2.3.3.4.1. Methane analysis. At the end of the incubation, the gas phase was analyzed for CH_4 using a micro-GC equipped with two gas chromatographic modules and a thermal conductivity detector (3000 micro-GC, Agilent, USA). Ethane (C_2H_6; 1 ml/flask) was used as the internal standard and argon as a carrier gas for both columns (Hassim et al., 2010).

2.3.3.4.2. Volatile fatty acids analysis. After opening the incubation flask, pH was measured (Hanna Instruments, Temse, Belgium), and 2 ml of incubation medium were collected and acidified with 200 µl of formic acid which contained the internal standard (10 mg 2-ethyl butyric acid/ml formic acid). After 15 min centrifugation at 4 °C and 22,000 × **g**, the supernatant was filtered with glass wool and an aliquot was transferred into a 1.5 ml glass vial. Samples were stored at 4 °C until VFA analysis using gas chromatography (Shimadzu 2010, Shimadzu Corporation, ‘s-Hertogenbosch, The Netherlands) equipped with a Nukol column (30 m × 0.25 mm × 0.25 µm, Supelco) with a flame ionization detector as described by Castro-Montoya et al. (2012).

2.3.3.4.3. Methane measurements for gas samples from the GPT technique. Using a gas tight syringe (Hamilton 1701 N, point style 5 needles, 51 mm; Hamilton, Bonaduz, Switzerland) an aliquot of 10 µl was sampled sequentially from the headspace gas of each bottle at 2, 4, 8, 12, 24, 30, 36, 48, 58 and 72 h and analyzed for CH_4. Methane was determined using a gas chromatograph (GC8000Top, CE Instruments, Milan, Italy) fitted to a flame ionization detector, using a packed column (PorapakQ, 6 m × 1/8 in., 50–80 mesh, Grace/Alltech, Lexington, Kentucky, USA) with nitrogen as carrier gas (100 kPa) and an oven temperature maintained at 60 °C (Pellikaan et al. 2011).

2.4. Statistical analysis

For the *in vivo* experiments the main effect of addition of essential oils was tested using the MIXED procedure of SAS with repeated measurements in time and using an autoregressive covariance structure according to:

$$Y_i = \mu + \beta_i + \xi_i$$

where μ=the overall mean; β_i=the effect of essential oils addition; and ξ_i=the error term. Orthogonal contrasts analysis was performed to look for differences between no-addition and addition of essential oils (Week 0 *vs*. Week 2–4–6) and polynomial contrasts were performed to look for significant linear and quadratic effects over time once essential oils were supplemented (Week 2–4–6). Significances were declared at $P<0.05$ and tendencies at $P<0.1$.

In experiment 1 (first series) of *in vitro* experiments the main effect of essential oils was tested by the GLM procedure of SAS software, according to:

$$Y_{ij} = \mu + \beta_i + \beta_j + \left(\beta_i \times \beta_j\right) + \xi_{ij}$$

where μ=the overall mean; β_i=the effect of the *i*th essential oils concentration; β_j=the effect of the *j*th substrate; ($\beta_i \times \beta_j$)=the interaction effect between substrate and concentration and ξ_i=the error term.

In experiment 2 the main effect of time of addition of essential oils was tested according to:

$$Y_i = \mu + \beta_i + \xi_i$$

where μ=the overall mean; β_i=the effect of time of addition; and ξ_i=the error term.

In experiments 3 (both series) and 4 the main effect of essential oils was tested by the GLM procedure of SAS software, according to:

$$Y_i = \mu + \beta_i + \xi_i$$

where μ=the overall mean; β_i=the effect of the *i*th essential oils; and ξ_i=the error term.

Effects of concentration of essential oils with $P<0.1$ were characterized using orthogonal contrasts testing the probability of linear responses. Coefficients for polynomial contrasts were calculated for the unequally spaced treatments using the ILM procedure of SAS software. Differences between means were evaluated using Tukey's multiple comparison test.

3. Results

3.1. In vivo experiments

3.1.1. Dairy cattle

Methane production data of week 4 are not presented for this experiment as they were unreliable in that period due to technical problems. All analysis and interpretations of this parameter are therefore based on results of week 0, 2, and 6. Table 3 shows daily milk production and milk composition. Milk production showed a linear decrease towards the end of the experiment. Whereas, fat, protein and lactose in milk did not show variations during the experimental period. Absolute daily enteric CH_4 production (g/d) and CH_4 relative to DMI (g/kg DM) and to milk production (g/kg milk) are presented in Table 4. Addition of a blend of essential oils tended to reduce CH_4 production in g/d and g/kg DM (P=0.07) as compared with the period without addition (week 0 vs. weeks 2 to 6). The decreases in CH_4 production accounted for 15% (g/d) and 14% (g/kg DMI) at the end of the experiment. These differences disappeared when CH_4 was expressed relative to milk production. However, a comparison in these units may be flawed due to the current experimental set-up in which the supplementation effect was assessed over time, which coincide with decreasing milk production due to progress in lactation (P=0.03, Table 3).

Furthermore, during the supplementation period (comparison of weeks 2 and 6) of Agolin Ruminant, a tendency (P=0.09) for a decrease in time of daily CH_4 production (g/d) was observed, this effect was not observed when CH_4 was expressed as g/kg milk.

Table 3
Milk production and milk composition of dairy cows supplemented with 0.2 g/d of essential oils during a six weeks experimental period. Week 0 represents the control (no essential oils supplementation).

	Week				SEM	*P* values		
	0 (0 g/d)	2 (0.2 g/d)	4 (0.2 g/d)	6 (0.2 g/d)		Week 0 vs. Week 2 to 6	Contrast[a]	
							L	*Q*
Milk production (kg/d)	19.5	19.8	17.9	16.9	0.727	0.21	0.03	0.50
Milk composition (g/100 g milk)								
Milk fat	4.75	4.80	4.80	4.80	0.093	0.71	0.79	0.82
Protein	3.41	3.28	3.50	3.59	0.077	0.78	0.04	0.05
Lactose	4.54	4.42	4.43	4.44	0.054	0.12	0.95	0.99

[a] Linear (*L*) and quadratic (*Q*) effects over time tested during the weeks 2 to 6, when the essential oils were supplemented.

3.1.2. Beef cattle

Absolute daily enteric CH_4 production (g/d), CH_4 relative to DMI (g/kg DM) and CH_4 relative to body weight (g/kg BW) are presented in Table 4. No differences were observed in daily CH_4 production, neither when CH_4 was corrected for DMI. However, when CH_4 was corrected for body weight a tendency to decrease CH_4 output was observed on addition of essential oils (week 0 vs. week 2–4–6; $P=0.09$). The reduction ranged between 13% and 20% (g CH_4/kg BW). During the supplementation period of essential oils there were no linear nor quadratic effects over time.

3.2. In vitro experiments

None of the concentrations tested in experiment 1 showed an effect on CH_4 formation ($P>0.10$) in combination with any of the two substrates (Fig. 1). Similarly, total VFA (ranging from 1742 to 1958 mmol/flask) and individual VFA proportions were not affected by the addition of the essential oils (data not shown). A substrate effect on CH_4 formation ($P<0.001$) was observed, with the Concentrate+Maize silage substrate producing more CH_4 than the Concentrate+Maize silage+Grass silage substrate, but differences disappeared when CH_4 was expressed relative to total VFA production. Moreover, there were no interaction effects of substrate and essential oils. Total VFA production was not affected by substrate type or interaction between substrate and essential oils ($P>0.10$). Given the absence of essential oils × substrate interactions all subsequent experiments were performed using the concentrate+grass silage+maize silage substrate.

In experiment 2, there was no difference for CH_4 formation and total VFA when essential oils were added one day prior to the start of the incubations or on the incubation day (Fig. 2).

In the first series of experiment 3, with sequential transfer every 24 h for a total incubation time of 96 h there was no effect of essential oils on CH_4 formation (Fig. 3A; $P<0.05$). Both for the control as well as the two essential oils concentrations, CH_4 formation strongly decreased after the first transfer and it kept on decreasing, albeit slightly, up to 96 h.

In the second series of experiment 3, where the transfer interval was extended to 48 h and the total incubation time was 14 days there was no effect of essential oils on CH_4 formation for any of the concentrations studied (Fig. 3B; $P<0.05$). The problem of

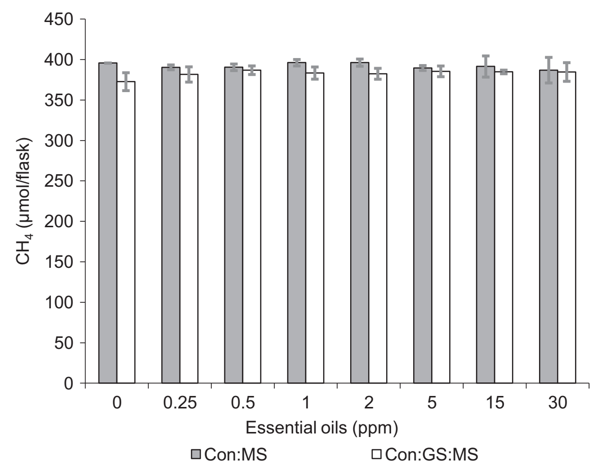

Fig. 1. *In vitro* methane (μmol/flask) production after 24 h batch incubation with essential oils. (Experiment 1). Conn:MS=Concentrate+Maize silage (50:50, DM basis); Conn:GS:MS=Concentrate+Grass silage+Maize silage (30:35:35, DM basis).

Table 4
Methane production of dairy cows or beef heifers supplemented with 0.2 g/d of essential oils during a six weeks experimental period. Week 0 represents the control (no essential oils supplementation).

	Week				SEM	*P* values		
	0 (0 g/d)	2 (0.2 g/d)	4 (0.2 g/d)	6 (0.2 g/d)		Week 0 vs. Week 2 to 6	Contrast[a]	
							L	*Q*
Dairy cattle								
g/d	283	252	–	241	9.07	0.07	0.09	–
g/kg DM	18.0	16.1	–	15.5	0.61	0.07	0.10	–
g/kg milk	14.6	13.1	–	14.9	1.71	0.64	0.19	–
g/kg BW	0.46	0.42	–	0.39	2.90	0.12	0.08	–
Beef cattle								
g/d	142	126	136	128	8.74	0.29	0.90	0.21
g/kg DM	17.1	15.1	16.3	15.3	1.06	0.23	0.90	0.20
g/kg BW	0.30	0.25	0.26	0.24	0.019	0.07	0.57	0.23

[a] Linear (*L*) and quadratic (*Q*) effects over time tested during the weeks 2 to 6, when the essential oils were supplemented.

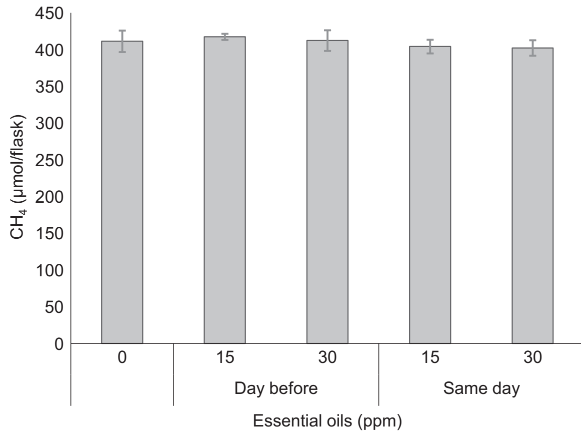

Fig. 2. *In vitro* methane (μmol/flask) and total VFA (mmol/flask) production after 24 h batch incubation with essential oils added one day before or on the same day of the incubation. (Experiment 2).

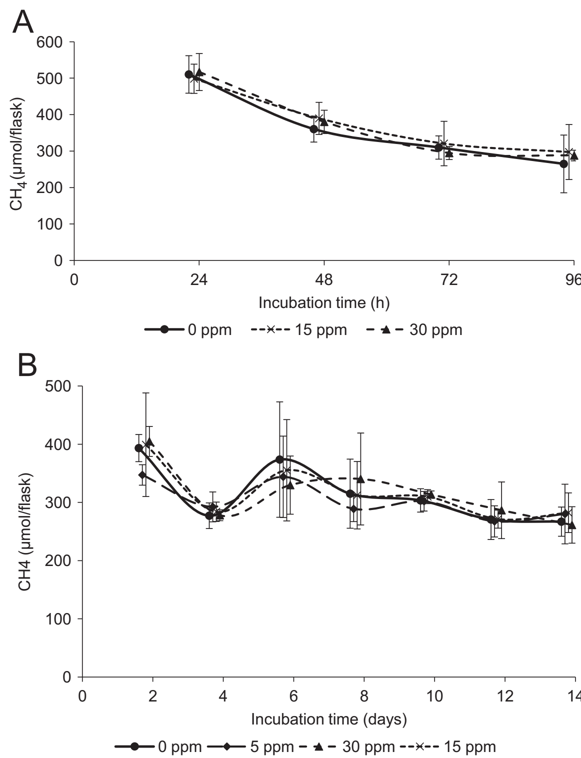

Fig. 3. *In vitro* methane (μmol/flask) production during consecutive batch incubations of 96 h with transfers every 24 h (A) and of 14 days with transfers every 48 h (B) with essential oils. (Experiment 3).

the continuous decrease in CH_4 production upon transfer had been avoided with the current experimental set-up.

In experiment 4, the gas production technique (GPT) was used to test for midterm effects (72 h) of essential oils on *in vitro* CH_4 formation (Fig. 4). Methane formation (ml/flask) decreased through addition of essential oils at 30 ppm after 30, 36 and 48 h and tended to decrease at 12, 24, 58 and 72 h (Fig. 4A). These decreases were accompanied by decreases in gas production (ml/flask) from 24 h onwards (tendencies at 58 and 72 h) (Fig. 4B). Hence, differences in CH_4 production disappeared when CH_4 was corrected for gas production (ml CH_4/ml gas), although tendencies for lower relative CH_4 production remained for the 30 ppm concentration after 12 and 48 h of incubation. Total VFA production after 72 h was not affected by the essential oils (3525, 3787 and 3408 mmol/flask for 0, 15 and 30 ppm, respectively; $P > 0.05$).

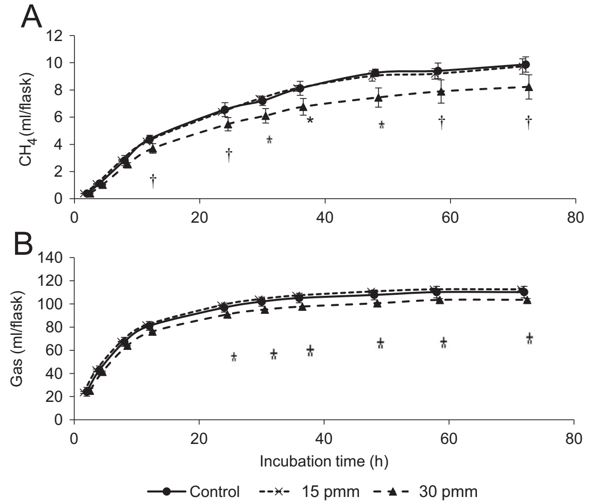

Fig. 4. Methane production (ml/flask, A) and total gas production (ml/flask, B) during 72 h incubation in a gas production technique with essential oils. (Experiment 4). Stars (*) indicate significant differences ($P < 0.05$) between treatments; crosses (†) indicate tendencies ($P < 0.1$).

4. Discussion

To our knowledge this is the first study reporting the effects of a blend of essential oils containing coriander oil, geranyl acetate and eugenol on CH_4 emissions from dairy and beef cattle combined with extensive *in vitro* experiments. Few *in vivo* studies have explored the effects of essential oils on CH_4 emissions. Moreover, these few studies showed inconsistent results, which are summarized in Table 5. Two studies with large ruminants showed opposite results: Beauchemin and McGinn (2006) did not find any effect of a blend of essential oils and spice extract (Crina Ruminants; Akzo Nobel Surface Chemistry S.A.) on CH_4 output, whereas Mohammed et al. (2004) reported a 19% decrease in CH_4 relative to DMI when supplementing the animals with horseradish oil. Two other studies with small ruminants again had contrasting results: Klevenhusen et al. (2011) did not find any effect of garlic oil on CH_4, while Wang et al. (2009) found that ropadiar decreased daily CH_4 by 12% relative to its control.

Nevertheless, comparisons across studies are disputable, as a replicated test with the same essential oil sources by different research groups has not been reported. Accordingly, differences in responses as reported here might relate to variation in composition in these products and the stability of their active compound. Diet is another factor differing between studies, as none of the experimental diets was the same.

Furthermore, in this study the essential oils dose equates to 12.8 and 24.2 mg/kg DMI for dairy and beef cattle, respectively, a dose which was 10 to 50 times lower than the doses provoking effects in previous studies (see Table 5). The current blend of essential oils decreased daily CH_4 emission in dairy cattle by 11% and 15% after 2 and 6 weeks of supplementation; although this effect was not observed in beef cattle. This lack of differences might be related to changes in body weight which were observed in beef cattle, who kept on gaining weight throughout the experimental period. Therefore larger animals were producing the same amount of CH_4, which is reflected on the decreases in CH_4 corrected by body weight ranging from 13% to 20% across the six weeks of supplementation.

It is interesting to notice that in both *in vivo* experiments the animals received the same dose of the essential oils, despite the obvious differences in body weight between dairy and beef cattle. In this study the commercial product supplying the blend of essential oils was fed to the animals at 1 g/d, delivering 0.2 g of

Table 5
Summary of previous *in vivo* studies supplementing essential oils to decrease methane emissions.

Author	(Source of) Essential oil	Animal	Basal diet[a]	Doses[b]			Effect on CH_4 (%)[c]		
				g/d	mg/kg BW	g/kg DMI	g/d	g/kg DMI	g/kg BW
Beauchemin and McGinn (2006)	Essential oils and spice extract[d]	Beef heifers	Barley silage (75%)+Steam rolled barley	1.0	3.85	1.39	+2.4	+7.8	n.r.
Mohammed et al. (2004)	Horseradish oil	Holstein steers	Sudangrass (60%)+Concentrate	2.74	19.4	0.7	n.r.	−19*	n.r.
Klevenhusen et al. (2011)	Garlic oil	Sheep	Hay (50%)+Concentrate	5.31	64.0	5.00	−9.0	−2.4	−7.9
Wang et al. (2009)	Ropadiar	Sheep	Hay (75%)+Concentrate	0.21	4.68	0.182	−12*	n.r.	n.r.

n.r.: Not reported.

[a] Roughage proportions (DM basis) reported between brackets.
[b] Doses of active compound(s).
[c] Change from the respective control treatment. Stars denote significant differences ($P < 0.05$) from the control.
[d] Mixture of thymol, limonene and guaiacol (Castillejos et al., 2005).

essential oils which equates to 0.33 and 0.40 mg/kg BW for dairy and beef cattle, respectively. The supplementation rate was according to the manufacturer recommendation for an adult animal. Knowing that dairy cattle were heavier and had a higher DMI, it could be expected that a similar dose would be less effective in dairy than in beef, however this was not the case, which might suggest that the effects of this blend of essential oils is not related to an acute effect on the rumen microbial population.

Essential oils are very diverse in composition, nature, active compounds and, hence, activity (Calsamiglia et al., 2007). Therefore, it might not be fair to compare the effect on CH_4 production of the blend used here with the effect shown by other essential oils. Nevertheless, it is still remarkable to reach decreases in CH_4 at such small doses. Furthermore, no adaptation of the rumen microorganisms to the blend of essential oils seemed to occur, as the product was able to reduce CH_4 emissions as soon as two weeks after starting its supplementation and, more importantly, these decreases were sustained over the 6 weeks of the supplementation.

For the dairy experiment, milk production decreased throughout the experimental period, and as a result CH_4 in g/kg milk returned to the levels of week 0 by the end of the experiment. The experiment was not designed to assess the effects of essential oils on milk production. However, this decrease was most likely the result of a normal decline in production due to lactation stage rather than a negative impact of the essential oils supplementation. Other milk components like fat, protein and lactose did not change when the blend of essential oils was supplemented.

In vitro screenings are commonly performed prior to testing products *in vivo*, although the poor relationship between *in vitro* and *in vivo* results is of major concern in this respect (Flachoiwsky and Lebzien, 2012). As concomitant *in vitro* and *in vivo* data are not largely available a second aim of this paper was to compare the effect of observed *in vivo* with *in vitro* observations using a variety of *in vitro* approaches which could be appropriate for screening purposes. A first series of test concentrations (experiment 1) included levels around the theoretical concentration of essential oils in the rumen when fed at the suggested dose. The dose of 0.2 g/d administered *in vivo* is equivalent to 2 to 4 mg/L, assuming rumen volumes of 50 L (beef heifers) and 100 L (dairy cows) and disregarding the passage rate, or 2–4 ppm (m/v). Furthermore, depending on the type of additive, there might be interaction effects between substrates and additives on CH_4 production (*e.g.* Castro-Montoya et al., 2012). As different diets were used during both *in vivo* experiments (dairy and beef), experiment 1 was performed using two substrates.

None of the concentrations used in experiment 1, had an effect on CH_4 or total VFA production, even though the highest concentration used was 15 times higher than the corresponding (based on assumptions) *in vivo* dose.

In previous studies with other essential oils *in vitro* CH_4 inhibition was achieved only at extremely high concentrations. Evans and Martin (2000) found that after 24 h thymol strongly inhibited *in vitro* CH_4 production when added at a concentration of 400 ppm, but production of acetate and propionate strongly decreased. When thymol was incubated at a concentration of 200 ppm or lower there were no effects on CH_4, acetate and propionate production. Similarly, Busquet et al. (2005) found that garlic oil and diallyl disulfide decreased *in vitro* CH_4 production (17 h incubation) when applied at a concentration of 300 ppm, but also total VFA production was decreased. Conversely, when both essential oils were added at lower concentrations (30 ppm) there was no negative effect on the fermentation, but no data on CH_4 production were reported.

The high concentrations used in our *in vitro* trials obviously increase the risk of impairing the fermentation and largely exceed the concentrations supplemented *in vivo* in the current study. Accordingly, knowing that the blend of essential oils was effective in decreasing CH_4 production at a theoretical concentration of 2–4 ppm (see above) one of the adjustments done on the methodology of the *in vitro* system was allowing the blend of essential oils to be in contact with feedstuffs and the environment from the afternoon before the start of the incubation. Such a procedure has been reported by Castillejos et al. (2005) in a study with Crina® (not reporting rumen CH_4 production). The normal practice *in vitro* is to add a freshly prepared solution of an additive shortly before the start of the simulation. Conversely, the regular practice *in vivo* is mixing the additive with the feed, generally the concentrate, prior to feeding, allowing some contact between the additive and the feed and exposure to the environment for some time before being ingested. However, the addition of essential oils the day before the incubation did not elicit any effect on CH_4 and total VFA production as compared with the addition shortly before the start of the fermentation.

Other types of *in vitro* incubations which could be used for screening purposes include prolonged incubation times which may allow to some extent to assess adaptation of rumen microbes to a certain product. The latter might be due to adaptation of the microbes or breakdown of the supplemented compound. On the other hand, it is possible that some products do not provoke acute effects at lower doses, but modification of the microbial population and changes in the fermentation may occur through longer exposure to these lower doses.

Within this study two methodologies were tested for a longer exposure time: a consecutive batch incubation approach and the gas production technique. First, in experiment 3, we used a

consecutive batch incubation technique, originally proposed by Theodorou et al. (1984) and recently used by Morgavi et al. (2013), to extend the incubation time avoiding the depletion of substrate and allowing for a daily supply of additive, as under *in vivo* conditions. It has been found that in the consecutive batch incubations protozoa tend to decline with each transfer and might not survive beyond a fifth transfer (Theodorou et al., 1984). However fibrolytic and sacharolytic bacteria, as well as methanogens do survive and proliferate in this system (Theodorou et al., 1987). Provided that the mode of action of essential oils is more related to their toxicity to gram-positive and gram-negative bacteria (Calsamiglia et al., 2007), and probably direct toxicity to archaea (Mohammed et al., 2004), than to any effect on protozoa, the incubation medium in this technique should still contain a representative microbial pool to test for the effects of essential oils on the fermentation and CH_4 production.

This more dynamic system would also allow for the growth of rumen microbes (only 5 ml aliquot from the previous incubation is transferred to the new one). After 3 transfers and 96 h incubation essential oils did not have any effect on CH_4 production when supplied daily at doses of 15 and 30 ppm. It is worth noticing that CH_4 production continuously decreased with each transfer, and seemed to stabilize between the incubations after 72 and 96 h. Such a decrease also has been observed by Morgavi et al. (2013). It might be possible that the decrease in CH_4 production in the control incubations through inoculum transfer every 24 h reflects a stress undergone by the microbial community, which may have masked the additive's effects.

Indeed, in the original study, Theodorou et al. (1984) reported that for 24 h transfers the fermentation stabilized only after the sixth transfer. Conversely, when transfers were done every 48 h and every 72 h the fermentation stabilized after 3 and 1 transfer, respectively. Taking this into account, in the second series of experiment 3, 48 h transfers were chosen. The latter were preferred over the 72 h transfers because these were thought to have a greater risk of depletion of the substrate. The 48 h transfers resulted in a more stable fermentation from the third transfer onwards, and gave the possibility to extend the whole incubation time up to 14 days, which corresponds with the first CH_4 registration *in vivo* after which CH_4 inhibition was observed. Additionally, a lower concentration of essential oils (5 ppm) was included in this experiment assuming that a longer incubation time could allow to observe effects of essential oils at lower concentrations. However, the addition of essential oils did not have any effect on CH_4 at any point of the incubation time and for none of the doses.

In experiment 4, using the GPT for 72 h, CH_4 and total gas production decreased when supplying the higher dose of essential oils from 30 and 24 h onwards, respectively. Although essential oils inhibited CH_4 production (17%) at a lower dose in the current GPT set-up, it may be surprising that this inhibitory effect already occurs after 30 h of incubation and CH_4 production already tended to be lower after 24 h of incubation, whereas essential oils at a dose of 30 ppm did not reduce methanogenesis in the 24 h batch incubation of experiments 1 and 2.

Some dissimilarities exist between both systems. The higher ratio of rumen fluid to buffer (1:2 vs. 1:4, for GPT and consecutive batch system, respectively) might lead to a faster depletion of the buffer and a concomitant decrease in pH, which might be inhibitory for some microbes (Getachew et al., 1998), and could have resulted in a higher sensitivity of microorganisms to essential oils in the GPT system.

Furthermore, microbial populations in rumen fluid from different species (sheep fed hay and cows fed grass silage, maize silage and concentrate, for batch systems and GPT, respectively) can have different capacities to degrade plant secondary compounds (Frutos et al., 2004; Kronberg and Walker, 1993), mycotoxins (Kiessling et al., 1984) and anti-nematodes/cestodes (Beretta et al., 1987). Other factors like collection time, diet of the donor animals and microbial community developed in each technique could have an influence in the different effects observed in both systems.

The inhibitory effects of essential oils observed *in vivo* were not replicated *in vitro*, stressing once again the differences between both systems and the need to make *in vitro* systems more reflective of *in vivo* conditions.

Essentially, the *in vitro* simulations used here assess a direct effect of the additive on microbial fermentative process taking place in the rumen. As outlined by Benchaar and Greathead (2011) there is little information about the fate of essential oils and their compounds in the gastro-intestinal tract. The mechanisms proposed for the disappearance of essential oil terpenes from the digestive tract include, among others, absorption across the intestinal wall into the blood system and later excretion in the urine (Michiels et al., 2008; Malecky et al., 2009), although these compounds may have provoked effects on the intermediary metabolism of the animal before being excreted. Eventually, *in vivo* depression of rumen CH_4 production trough essential oils was indirect through a host-microbe interaction. These effects are not simulated through the current *in vitro* set-up and potentially should be emphasized in the future.

5. Conclusions

The blend of essential oils tended to decrease daily CH_4 emissions from dairy and CH_4 emissions corrected by body weight in beef cattle at a low dose compared with doses of other essential oils as reported in literature and the decrease was sustained for the six weeks of supplementation. However, none of the *in vitro* systems studied showed this inhibitory effect at such low doses. Only the GPT system, run over 72 h, showed a similar decrease in CH_4 but only at some incubation times and at doses which were 15-fold greater as compared with the *in vivo* effective dose. If *in vitro* batch incubations are to be used as screening tools for additives to decrease CH_4 production, the system still needs to be improved in order to avoid ruling out promising products based only on their limited effects observed *in vitro*.

Conflict of interest

I, Joaquín Castro-Montoya, on behalf of all co-authors state that there is no conflict of interests related to this manuscript.

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