Effects of essential oils from African basil on fermentation of *Andropogon gayanus* grass in the Artificial Rumen (RUSITEC)

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[1]*Agriculture and Agri-Food Canada, Lethbridge Research Centre, 5403 1st Anvenue South, Lethbridge, Alberta, Canada T1J 4B1; and* [2]*Laboratoire de Pharmacognosie et des Huiles Essentielles, Faculté des Sciences et Techniques, Faculté des Sciences de la Santé, Université d'Abomey-Calavi, ISBA Champ de Foire, 01 BP 918, Cotonou, Benin.*

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Kouazounde, J. B., Gbenou, J. D., He, M., Jardim, T., Jin, L., Wang, Y., Beauchemin, K. A. and McAllister, T. A. 2015. **Effects of essential oils from African basil on fermentation of *Andropogon gayanus* grass in the Artificial Rumen (RUSITEC)**. Can. J. Anim. Sci. **95**: xxx–xxx. Essential oils (EO) from African basil (*Ocimum gratissimum*) have shown the potential to modify rumen microbial fermentation and reduce ruminal methane production from grass forages in in vitro batch cultures. However, it is not known whether the effects of EO on rumen microbial fermentation attenuate over time. The objective of this study was to examine the effects of African basil EO at 0 (control), 100, 200 and 400 mg L^{-1} incubation medium on microbial fermentation and methane production in the Rumen Simulation Technique (RUSITEC) using *Andropogon gayanus* grass as a substrate. African basil EO quadratically affected ($P<0.05$) methane production gas production and the pH of fermenter liquid. Total volatile fatty acid (VFA) production was linearly decreased ($P<0.05$) by African basil EO along with a shift in VFA profile towards less propionate and more acetate and butyrate. African basil EO quadratically altered ($P<0.05$) apparent dry matter, neutral detergent fiber digestibility, ^{15}N incorporation into total microbial protein and the total production of microbial protein. This study confirms that EO from African basil quadratically affected methane emissions arising from the ruminal fermentation of *A. gayanus* grass mainly by reducing overall digestibility of the forage.

Key words: African basil, essential oils, tropical grass, rumen simulation technique, methane, digestibility

Kouazounde, J. B., Gbenou, J. D., He, M., Jardim, T., Jin, L., Wang, Y., Beauchemin, K. A. and McAllister, T. A. 2015. **Les effets des huiles essentielles du basilic africain sur la fermentation de l'herbe *Andropogon gayanus* dans le rumen artificiel (RUSITEC)**. Can. J. Anim. Sci. **95**: xxx–xxx. Les huiles essentielles (EO – « essential oils ») provenant du basilic africain (*Ocimum gratissimum*) montrent un potentiel pour modifier la fermentation microbienne dans le rumen et réduire la production de méthane ruminal à partir de fourrages d'herbes dans les cultures en batch in vitro. Cependant, il n'est pas connu si les effets des EO sur la fermentation microbienne dans le rumen s'atténuent avec le temps. Les objectifs de cette étude étaient d'examiner les effets des EO du basilic africain à 0 (témoin), 100, 200 et 400 mg L^{-1} de milieu d'incubation sur la fermentation microbienne et la production de méthane dans le simulateur technique du rumen (RUSITEC) en utilisant l'herbe *Andropogon gayanus* comme substrat. Les EO du basilic africain ont eu un effet quadratique ($P<0,05$) sur la production de méthane, la production de gaz et le pH du liquide à fermentation. La production d'acides gras volatils (VFA – « volatile fatty acids ») totaux diminuait de façon linéaire ($P<0,05$) par les EO du basilic africain et il y avait une transition dans le profil des VFA vers moins de propionate et plus d'acétate et de butyrate. Les EO du basilic africain modifiaient de façon quadratique ($P<0,05$) les matières sèches (DM – « dry matter ») apparentes, la digestibilité des fibres de détergent neutre (NDF – « neutral detergent fibre »), l'incorporation du ^{15}N dans les protéines microbiennes totales et la production totale de protéines microbiennes. Cette étude confirme que les EO du basilic africain ont un effet quadratique sur les émissions de méthane provenant de la fermentation ruminale de l'herbe *A. gayanus* principalement par réduction de la digestibilité globale du fourrage.

Mots clés: Basilic africain, huiles essentielles, herbe tropicale, technique de simulation du rumen, méthane, digestibilité

African basil [*Ocimum gratissimum* L. (Labiatae)] is an aromatic herbaceous plant native to tropical and subtropical regions and is abundant in India and West Africa. This plant is widely used in folklore medicine for its antiseptic properties and the treatment of digestive disorders including stomach upset and diarrhea (Prabhu et al. 2009). *Ocimum gratissimum* contains various plant secondary metabolites including tannins, essential oils

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Abbreviations: DM, dry matter; **DMD**, apparent DM disappearance; **EO**, essential oils; **FPA**, feed particle-associated; **FPB**, feed particle-bound; **GP**, gas production; **NDF**, neutral detergent fiber; **NDFD**, NDF disappearance; **RUSITEC**, rumen simulation technique; **VFA**, volatile fatty acid

(EO), saponins and flavonoids that are known to influence microbial activity, rumen microbial fermentation and enteric methane production (Prabhu et al. 2009; Bodas et al. 2012).

Recent in vitro batch culture studies conducted in our laboratory revealed that EO from *O. gratissimum* at 200 to 400 mg L^{-1} altered rumen microbial fermentation of *Andropogon gayanus* Kunth (Poaceae) grass and reduced ruminal production of methane by up to 30% [per gram dry matter (DM) incubated] relative to control (Kouazounde et al. 2014). *Andropogon gayanus* (gamba grass) is a tall, tufted perennial grass native to the tropical and subtropical savannas of Africa and has the ability to tolerate long dry seasons and low-fertility, acidic soils. It was introduced to many other tropical areas of the world, including the tropics of America, India, and Western Australia owing to its high biomass production (Ahmed 1990).

Discrepancies have been previously documented in responses observed with plant extracts in short-term in vitro batch cultures versus continuous culture or in vivo studies (Busquet et al. 2005; Benchaar and Greathead 2011). These differing responses may be due to the ability of microbial populations to adapt to and/or degrade a wide variety of plant secondary metabolites including EO over time (Busquet et al. 2005; Benchaar et al. 2008; Benchaar and Greathead 2011). Therefore, in this study, we assessed the effects of EO from *O. gratissimum* acclimated to Benin (West Africa) on rumen microbial fermentation of *A. gayanus* grass using the rumen simulation technique (RUSITEC).

MATERIALS AND METHODS

Essential Oil

The EO from *O. gratissimum* was obtained from the Laboratory of Pharmacognosy and Essential Oils of the University of Abomey Calavi, Benin. It was extracted from plant leaves by steam-distillation using a Clevenger-type apparatus and analyzed by gas chromatography coupled to mass spectrometry (GC-MS) as described by Kouazounde et al. (2014). The main components in *O. gratissimum* EO are γ-terpinolene (175.2 g kg^{-1}), p-cymene (199.5 g kg^{-1}) and thymol (275.6 g kg^{-1}) (Kouazounde et al. 2014).

Experimental Design and Treatments

Effects of *O. gratissimum* EO on ruminal fermentation of *A. gayanus* were assessed at various doses in two identical and concomitant incubation runs using an eight-vessel RUSITEC system (Czerkawski and Breckenridge 1977). The two incubation runs were carried out independently and treatments were designed randomly with two fermenters receiving a single treatment per run, so that each treatment had four replicates. Treatments were control (no EO), 100, 200 and 400 mg L^{-1} incubation medium of *O. gratissimum* EO. Dosages of *O. gratissimum* EO were selected based on Kouazounde et al. (2014), who reported reductions in methane production with *O. gratissimum* EO at 200 to 400 mg L^{-1}. Incubation runs lasted 16 d and consisted of a 7-d adaptation period to enable fermenters to achieve steady-state conditions followed by a treatment and measurement period of 9 d.

Substrate, Source of Inoculum, Incubation Procedure

A mixture of ground *A. gayanus* grass and supplement containing minerals and vitamins was used as the substrate. The substrate consisted of grass and supplement in a ratio of 95:5 [wt/wt, dry matter (DM) basis]. The forage was collected by cutting aerial plant parts 10 cm above the soil surface at the flowering stage at the peak of the dry season in July and August 2013 at the pilot farm of the Faculty of Agronomic Science, Benin (6.2°N, 2.2°E). Grass samples were ground through a 4-mm screen and pooled in a single lot after 3 d of sun-drying followed by 2 d of oven-drying at 60°C.

The inoculum including rumen fluid and solid digesta was collected from two ruminally fistulated non-lactating cows fed a 25:75 barley grain:barley silage diet. Cows were cared for in accordance with standards set by the Canadian Council on Animal Care (2009). Rumen fluid and solid digesta were obtained by straining rumen contents collected from four sites within the rumen 2 h after the morning feeding through four layers of sterilized cheesecloth into pre-warmed insulated containers (39°C). Approximately 200 g of the solid digesta were collected into a pre-warmed insulated container (39°C) for initial inoculation of the fermenters. Collected rumen fluid and solid digesta were transported immediately under anaerobic conditions to the laboratory, where rumen fluid samples were pooled in equal portions.

RUSITEC systems were operated in a manner similar to that previously described by Li et al. (2013). Fermenters were inoculated with 180 mL of artificial saliva (McDougall's 1948) modified to contain 1 g L^{-1} of incubation medium, 720 mL of strained rumen fluid and two nylon bags (10 × 20 cm, mean pore size 50 μm, ANKOM, Macedon, NY), one containing 20 g of fresh hand-squeezed rumen solid digesta and the second 10 g of substrate. After 24 h, the bag with solid digesta was replaced with a new feedbag, and thereafter the 48-h feedbags were replaced daily, such that two bags containing feed were present at any one time.

From day 8 to the end of the experiment, the EO was dissolved in ethanol (volume of ethanol = 2 mL − EO volume) and added directly into the fermentation fluid of the treatment vessels. An equivalent volume of ethanol (2 mL) without EO was added to control fermenters.

Sampling and Analysis

Fermenter pH, gas production (GP) and effluent volumes from each fermenter were measured daily at the time of feeding over the 16 d of the study. The daily GP was collected and measured as described by Fraser et al.

(2007). A pH meter (Orion model 260A, Fisher Scientific, Toronto, ON) was used to measure the pH of the fermenter liquid. Effluent was collected in 2-L flasks prefilled with 5 mL of aqueous solution of sodium azide (1 g L^{-1}) to stop microbial fermentation. From day 8 to day 16, collected effluent was sampled at the time of feeding and processed for volatile fatty acid (VFA) and ammonia analysis as described by Wang et al. (1998). Prior to gas measurement on days 8–16, a 10-mL sample of gas was obtained from the septum of collection bags using a 26-gauge needle (Becton Dickinson, Franklin Lakes, NJ) and transferred to evacuated 6.8-mL exetainers (Labco Ltd., Wycombe, Bucks, UK) for later analysis of daily methane concentration (Chaves et al. 2006; Fraser et al. 2007). The 48-h feedbags removed from each fermenter were processed to determine apparent DM disappearance (DMD) from day 8 to the end of the experiment as described by Li et al. (2013). Dried residues from feedbags removed on the last 4 d were ground through a 1-mm screen and analyzed for neutral detergent fiber (NDF) according to Van Soest et al. (1991) to determine NDF disappearance (NDFD) with heat-stable α-amylase and sodium sulphite being used in the NDF procedure and expressed inclusive of residual ash.

Microbial protein synthesis was estimated using the ^{15}N marker procedure. A portion of the $(NH_4)_2SO_4$ in McDougall's buffer solution was replaced with ^{15}N-enriched $(NH_4)_2SO_4$ (Sigma Chemical Co., minimum ^{15}N enrichment 1 g L^{-1}) from day 8 until the end of the experiment as described by Li et al. (2013). The background ^{15}N was determined from a volume of 250 mL of effluent (preserved in a solution of azide) sampled from each collection flask and the removed 48-h feedbags on day 8, before the addition of ^{15}N-enriched $(NH_4)_2SO_4$ to the McDougall's buffer solution. The ^{15}N enrichment in non-ammonia nitrogen was determined from the preserved effluent (250 mL) sampled from each collection flask and the removed 48-h feedbags on days 14 and 15. The collected feedbags were processed to obtain feed particle-associated (FPA) and feed particle-bound (FPB) bacterial fractions as described by Wang et al. (2001). Feedbags were gently squeezed to expel excess liquid, then placed in a plastic bag with 20 mL of McDougall's buffer, and processed for 60 s in a Stomacher 400 laboratory blender (Seward Medical Ltd., London, UK). The processed liquid was squeezed out and retained, and the feed residues were washed twice with 10 mL buffer per wash. The two 10-mL washings were pooled with the initially expressed liquid to obtain the FPA bacterial fraction, and the total volume was recorded. The washed feed residues, representing the FPB bacterial fraction, were retained. All samples were stored at − 20°C until analysed.

The collected effluents samples (fractions of liquid-associated), FPA and FPB bacterial fractions were analyzed for DM, ^{15}N and total N by combustion analysis (NA 1500, Carlo Erba Instruments, Rodano, MI, Italy), and used to estimate microbial nitrogen production as described by Wang et al. (2001). Effluent liquid samples and FPA bacterial samples collected from the stomaching process were centrifuged (20 000 × *g*; 30 min; 4°C), and the resulting pellets were washed with 7 M phosphate buffer (pH 7.2) and centrifuged (20 000 × *g*; 30 min; 4°C) three times. The pellet was then resuspended in 5.0 mL water, combined with 1.0 mL 5% (wt/vol) NaOH and dried at 55°C. The FPB bacterial samples were resuspended in 10 mL buffer, and washed, dried and treated with NaOH as per liquid samples. Dried materials were weighed for DM determination and ball milling with a Pulverisette 7 planetary micro-mill (Laval Lab Inc., Laval, QC) for measurement of total N and ^{15}N enrichment.

The substrate was analyzed for DM and organic matter content (Association of Official Analytical Chemists 2003), NDF and acid detergent fiber as described by Van Soest et al. (1991). Crude protein was estimated as N × 6.25 with N being measured by combustion analysis (NA 2100, Carlo Erba Instruments, Rodano, Milan, Italy). The nutrient composition of the substrate used in the current study was as follows (g kg^{-1} DM): organic matter (902.6), NDF (678.7), acid detergent fiber (424.9) and crude protein (89.3).

Calculations and Statistical Analysis

The 48-h DMD and NDFD were calculated as the difference between incubated DM and NDF weight of the substrate and those of the residue from the removed 48-h feedbags, respectively. Total VFA, methane and GP and ammonia-N in the incubation fluid were estimated per day of incubation. The mean values of these parameters, as well as DMD and NDFD, were estimated over the last 4 d to have a single value per fermenter per run, and these values were used as the experimental unit for statistical analyses.

Microbial protein synthesis in effluent and FPA fractions produced daily were calculated from the weight of their associated microbial pellet isolated and the total N content. Daily microbial protein in the FPB fraction was calculated from ^{15}N enrichment of the microbial pellet isolated from the FPA fraction and total N content. ^{15}N enrichment of the FPB fraction was estimated as suggested by Wang et al. (2000). The total microbial protein synthesis per day was estimated for each fermenter as the sum of the microbial protein measured in its associated individual fractions (effluent, FPA, and FPB). The mean values of microbial protein synthesis in effluent, FPA and FPB fractions and the total microbial protein synthesis were estimated over the last 2 d to have a single value per fermenter per run, and these values were used as the experimental unit for the statistical analyses.

All data were analyzed using PROC MIXED (SAS Institute Inc. 2012) with the EO treatment in the model as a fixed effect and run as a random effect. PROC UNIVARIATE (SAS Institute Inc. 2012) was used to test residuals for normality. Outlying data that were more

than two standard deviations from the mean were removed before final analysis. Differences among least squared means were only compared after a protected F test using LSD, and orthogonal polynomials were also fitted to test for linear and quadratic responses to the four dosages. Differences were declared significant at $P<0.05$.

RESULTS

Incubation medium pH, total GP and methane production were quadratically affected ($P<0.05$), whereas production of total VFA was linearly decreased ($P<0.05$) by *O. gratissimum* EO (Table 1). Acetate and the acetate to propionate ratio were linearly increased ($P<0.05$) by *O. gratissimum* EO, whereas propionate, butyrate, branch-chained VFA and valerate responded quadratically ($P<0.05$). The DMD was quadratically affected ($P<0.05$), while NDFD was linearly decreased ($P<0.05$) by *O gratissimum* EO.

Addition of *O. gratissimum* had no effect ($P>0.05$) on ammonia-N concentration (Table 2). Total incorporation of ^{15}N into microbial protein and total microbial protein and their associated effluent and FPA fractions responded quadratically ($P<0.05$) to increasing *O. gratissimum* EO. Likewise, microbial protein and ^{15}N enrichment of the FPB fraction also responded qadratically ($P<0.05$) to increasing *O. gratissimum* EO.

Total microbial incorporation of ^{15}N and total microbial protein, and their associated individual fractions (effluent, FPA, and FPB) were quadratically affected ($P<0.05$) by *O gratissimum* EO (Table 2).

DISCUSSION

Methane production was affected by *O. gratissimum* EO in a dose-dependent manner as evidenced by the quadratic response ($P<0.05$) to EO dosage. It is well known that inhibition of methane production may result from an increase in propionate acting as an alternative electron acceptor and a decrease in overall fermentation (Janssen 2010; Bodas et al. 2012). However, an increase in acetate and butyrate production has been observed to promote methane production (Moss et al. 2000). The observed quadratic response of methane to *O. gratissimum* EO dosage may be explained by the antagonistic effects on methane production resulting from the increase in acetate and butyrate and the decline in substrate fermentation as evidenced by the decrease in DMD and total VFA.

In agreement with the present study, *O. gratissimum* EO was recently shown in an in vitro batch culture study to quadratically affect methane production, primarily through a reduction in overall rumen fermentation of *A. gayanus* grass (Kouazounde et al. 2014). Similarly, Benchaar and Greathead (2011) reported in their review that thymol and *Thymus vulgaris* (rich in thymol), at over 200 mg L^{-1}, inhibited methane production from a 25:75 mixed forage–concentrate diet in batch cultures. They also reported that this response was associated with an overall reduction in rumen fermentation, even at lower dosages. Patra and Yu (2012) also observed that, at 250 to 1000 mg L^{-1}, *Thymus capitatus* linearly decreased methane production owing to a reduction in digestibility of an alfalfa hay:concentrate mixture (50:50) in vitro batch cultures.

The linear decrease in total VFA concentration with addition *O. gratissimum* EO was consistent with the concomitant increase in pH of the incubation medium and the decrease in GP, suggesting a reduction in rumen microbial fermentation of *A. gayanus* grass. There have been no other studies done using a RUSITEC system to evaluate the effects of *O. gratissimum* EO on total VFA or individual VFA. In agreement with the effects

Table 1. Effect of the essential oils from *Ocimum gratissimum* on fermentation of *Andropogon gayanus* grass in the rumen simulation technique (RUSITEC)

		Ocimum gratissimum (mg L^{-1})				Main effects, *P* value		
Parameter[z]	Control	100	200	400	SEM	Control vs. EO	L	Q
pH	6.8*d*	6.9*c*	7.1*b*	7.3*a*	0.01	<0.001	<0.001	<0.001
Total gas (mL d^{-1})	1077.2*a*	1016.7*ab*	943.8*b*	578.8*c*	32.69	<0.001	<0.001	0.001
Methane (mL d^{-1})	8.6*a*	12.0*a*	13.6*a*	1.8*b*	2.12	0.011	0.073	0.004
Total VFA (mmol d^{-1})	44.0*a*	36.3*b*	16.5*c*	5.8*d*	1.13	<0.001	<0.001	0.203
VFA (mol 100 mol^{-1})								
Acetate	56.8*b*	59.6*ab*	61.0*a*	61.7*a*	1.16	0.052	0.009	0.384
Propionate	35.1*a*	28.5*b*	13.8*c*	11.3*c*	0.93	<0.001	<0.001	0.045
Butyrate	6.9*d*	10.6*c*	17.8*b*	26.5*a*	0.92	<0.001	<0.001	0.022
Valerate	0.5*d*	0.8*c*	1.6*a*	1.0*b*	0.06	<0.001	<0.001	<0.001
BCFVA	0.6	0.6	0.5	0.6	0.05	0.157	0.854	0.048
Acetate: propionate	1.6*b*	2.1*b*	4.7*a*	5.5*a*	0.29	<0.001	<0.001	0.553
NDFD – 48 h (g kg^{-1})	324.8*a*	253.3*b*	166.0*c*	47.8*d*	11.28	<0.001	<0.001	0.061
DMD – 48 h (g kg^{-1})	396.0*a*	352.7*b*	302.1*c*	206.5*d*	7.04	<0.001	<0.001	0.003

[z]VFA, volatile fatty acid; BCVFA, branched-chain volatile fatty acid; DMD, apparent dry matter disappearance; NDFD, apparent neutral detergent fiber disappearance; EO, essential oils from *Ocimum gratissimum;* L, linear contrast; Q, quadratic contrast.
a–d Mean values for an additive with different letters in the same row differ significantly ($P<0.05$).

Table 2. Effect of the essential oils from *Ocimum gratissimum* on production of NH_3-N, incorporation of ^{15}N into microbial N in effluent, feed particle-associated (FPA), and feed particle-bound (FPB) fractions from the fermentation of *Andropogon gayanus* in the rumen simulation technique (RUSITEC)

		Ocimum gratissimum (mg L^{-1})				Main effects, *P* value		
Parameter[z]	Control	100	200	400	SEM	Control vs. EO	L	Q
NH_3-N (mg d^{-1})	6.8	7.1	6.5	7.2	0.28	0.337	0.612	0.802
Total microbial incorporation of ^{15}N (mg 48 h^{-1})	1.7*a*	1.7*a*	1.6*a*	0.73*b*	0.05	<0.001	<0.001	<0.001
Effluent (μg d^{-1})	641.4*a*	526.8*ab*	500.9*b*	144.6*c*	37.63	<0.001	<0.001	0.002
FPA (μg 48 h^{-1})	602.5*a*	642.0*a*	468.7*b*	121.5*c*	23.17	<0.001	<0.001	<0.001
FPB (μg 48 h^{-1})	411.6*c*	498.0*b*	645.9*a*	466.5*bc*	18.66	<0.001	0.001	0.002
Total production of MP (mg 48 h^{-1})	247.0*a*	221.3*a*	186.3*b*	140.9*c*	8.44	<0.001	<0.001	0.016
Effluent (mg d^{-1})	66.4*a*	52.2*b*	49.1*b*	17.2*c*	3.79	<0.001	<0.001	0.005
FPA (mg 48 h^{-1})	107.2*a*	95.1*a*	57.7*b*	25.6*c*	4.98	<0.001	0.001	0.007
FPB (mg 48 h^{-1})	73.4*b*	74.1*b*	79.4*b*	98.1*a*	2.87	0.001	0.001	<0.003

[z]FPA, feed particle-associated; FPB, feed particle-bound; EO, essential oils from *Ocimum gratissimum;* L, linear contrast; Q, quadratic contrast.
a–c Mean values for an additive with different letters in the same row differ significantly ($P<0.05$).

of *O. gratissimum* EO on VFA production in the present study, Castillejos et al. (2006) observed that thymol (500 mg L^{-1}) decreased total VFA concentration and increased butyrate in a dual-flow continuous culture system fed a 60:40 forage:concentrate diet. However, they also reported that thymol (500 mg L^{-1}) decreased the proportion of acetate and the acetate:propionate ratio owing to an increase in propionate. Thymol from *T. capitatus* has been shown to increase pH in in vitro batch cultures with both alfalfa hay and an alfalfa hay:concentrate mixture (50:50), which was accompanied by a decrease in total VFA concentration and GP (Kamalak et al. 2011; Patra and Yu 2012). Effects exhibited by *O. gratissimum* EO on VFA profile in the current study are in agreement with Patra and Yu (2012), who observed that *T. capitatus* shifted the VFA profile towards less propionate and more acetate and butyrate. In contrast, Kamalak et al. (2011) noted that addition of thymol up to 200 mg L^{-1} had no effect on molar proportions of acetate, propionate or butyrate. Kouazounde et al. (2014) reported that increasing doses of *O. gratissimum* EO linearly decreased total VFA concentration and GP from *A. gayanus* grass in batch cultures.

Addition of *O. gratissimum* EO consistently decreased DMD (quadratic effect; $P<0.05$) with a reduction in NDFD, total VFA concentration, GP (DM basis) and incorporation of ^{15}N into microbial protein, suggesting that this EO exerts a non-specific inhibitory effect on rumen microbial activity. The reduction in NDFD suggests that cellulolytic bacteria were particularly sensitve to *O. gratissimum* EO, an effect that was likely responsible for the observed decrease in DMD. Microbial attachment plays a key role in the digestion of fiber by cellulolytic bacteria (McAllister et al. 1994). In this study, *O. gratissimum* EO also decreased microbial attachment as evidenced by a reduction in the incorporation of ^{15}N into FPA microbial fraction and a decline in the production of FPA microbial protein. Castillejos et al. (2006) also reported that thymol at 500 mg L^{-1} decreased DMD, NDFD and total VFA concentration in a dual-flow continuous culture fed a 60:40 forage:concentrate diet. Hyldgaard et al. (2012) and Ngassoum et al. (2003) used the agar disk diffusion method to show that EO from *O. gratissimum* exhibited broad-spectrum antimicrobial activity against both Gram-negative and Gram-positive bacteria. The antimicrobial activity of thymol is thought to arise as a result of increased permeability of the bacterial cell membrane (Hyldgaard et al. 2012), an outcome consistent with the high thymol content of *O. gratissimum* (Dorman and Deans 2000; Burt 2004; Hyldgaard et al. 2012). Gamma terpinolen and p-cymene are also abundant in *O. gratissimum* and may also contribute to the antimicrobial properties of the EO extract, as they have been shown to inhibit both ruminal and non-ruminal bacteria (Oh et al. 1967; Dorman and Deans 2000). Although the antimicrobial activity of p-cymene is limited, when combined with thymol antimicrobial activity is enhanced, possibly by it facilitating thymol transport across the cell membrane (Benchaar and Greathead 2011). Patra and Yu (2012) reported that enriched thymol EO from *T. capitatus* at 250 to 1000 mg L^{-1} decreased DMD of a 50:50 ground alfalfa hay:concentrate mixture, resulting in a decline in total VFA and GP. *Thymus zygis*, which contains mainly thymol (62.1%), also reduced DMD of a 30:70 alfalfa hay:barley grain mixture when included in batch cultures (Martinez et al. 2006). Finally, Kamalak et al. (2011) observed a decrease in true DMD of alfalfa hay with a reduction in true NDFD, total VFA and GP when 50 to 200 mg L^{-1} of thymol was added to batch cultures.

CONCLUSION

Supplementation of EO from African basil exerted a quadratic effect on methane production by altering rumen microbial fermentation of *A. gayanus* grass in the RUSITEC, thereby lowering digestibility, total VFA and microbial protein synthesis, and shifting the VFA profile towards less propionate and more acetate and butyrate. This study confirms previous results from batch fermentation systems that the quadratic effect on methane from

low-quality grass forage supplemented with EO from *O. gratissimum* was mainly due to a reduction in digestibility. The adverse effect of *O. gratissimum* EO on in vitro digestibility and total VFA production suggests that it would likely broadly lower rumen microbial fermentation in vivo through a reduction in diet digestibility, an outcome that would reduce production efficiency making such an approach undesirable even if it were to lower methane emissions.

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