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Assessment of Rumen Microbial Adaptation to Garlic Oil, Carvacrol and Thymol Using the Consecutive Batch Culture System

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Abstract

Although plant derivatives have shown promise in reducing enteric methane (CH_4) emissions from ruminants in short-term studies, investigation on possible rumen microbial adaptation to these compounds is still limited. The objective of this study was to assess the possibility of mixed rumen microbial adaptation to antimethanogenic plant derivatives over relatively long-term i*n vitro* incubation. Treatments were: garlic oil, carvacrol and thymol, each at a final concentration of 300 mg/l. A control, containing no additive, was included. The fermentations were done using the consecutive batch culture system with two serial subculture transfers at three-day intervals. The substrate was a mixture of forage and concentrate in a ratio of 60:40. Incubations were conducted in triplicate with rumen fermentation parameters being determined at days 3, 6 and 9. Indications of rumen microbial adaptation were observed in carvacrol and thymol as CH_4 significantly increased from almost complete inhibition at day 3 to almost 5 % of total gas at day 9. This was closely associated with a rise in methanogenic archaea populations from being significantly lower ($p = 0.0026$) at day 3 to being numerically higher ($p = 0.0632$) than the control by day 9. Garlic oil consistently suppressed ($p < 0.0001$) CH_4. Carvacrol and thymol suppressed overall fermentation, resulting in lower ($p < 0.0001$) VFA concentrations and hence higher culture pH ($p < 0.0001$). Garlic oil did not differ from the control on VFA concentrations ($p > 0.05$) except for butyrate ($p = 0.04$). Treatments had no overall effect on total bacteria ($p = 0.235$) and protozoa numbers ($p = 0.835$). The effects of carvacrol and thymol on fermentation parameters suggested that these supplements lower CH_4 indirectly by suppressing overall fermentation whereas garlic oil selectively inhibits methanogens directly. We concluded that rumen microbes adapted to carvacrol and thymol. These supplements need to be tested *in vivo* to see if similar effects will be observed.

Keywords: Consecutive batch culture; Essential oils; Methane; Microbial adaptation; Rumen fermentation

Abbreviations: A/P: Acetate : Propionate; CH_4: Methane; DM: Dry matter; DNA: Deoxyribonucleic acid; H_2: Hydrogen; N: Nitrogen; PCR: Polymerase chain reaction; qRT-PCR: Quantitative real-time polymerase chain reaction; rRNA: Ribosomal ribonucleic acid; VFA: Volatile fatty acid

Introduction

Livestock production, particularly ruminant production, contributes significant amounts of methane (CH_4), a greenhouse gas with a global warming potential at least 20 times that of carbon dioxide [1], into the atmosphere. CH_4 originating directly from ruminants is a byproduct of carbohydrate fermentation. Ninety percent of enteric CH_4 from ruminant animals comes from the fore stomach and 10 % comes from hindgut fermentation [2]. Recent efforts to reduce CH_4 emissions from ruminants have focused on natural products, primarily of plant origin. Although plant-based products have generated a lot of attention, relatively fewer long-term studies on their effects have been conducted to date. Most *in vitro* studies on the effects of essential oils and other plant products have been short-term incubations, lasting 24 h to 48 h [3-5]. This poses a challenge considering that rumen microbial adaptation to these plant-based products remains a possibility over longer periods of exposure [6,7].

Carvacrol and thymol are related phenolic compounds found in essential oils of thyme and oreganum [8]. These compounds have long been established as antibacterial and antifungal. They have been shown to affect a wide range of Gram-positive and Gram-negative bacteria [9]. Although they reduce CH_4 production *in vitro*, their incorporation also reduces volatile fatty acid (VFA) concentration, hence would be deleterious to animals if these effects are sustained *in vivo* [3]. Suppressed VFA concentrations are a result of overall fermentation being negatively affected. However, Busquet *et al.* [7], after applying carvacrol at a low dose (2.2 mg/l) in a continuous culture system suggested that rumen microbes could adapt to carvacrol. Studies in food preservation have also shown that pathogenic bacteria ordinarily inhibited by carvacrol or thymol can adapt to non-lethal concentrations of these phenolic compounds [8,10].

Garlic oil, a derivative of garlic, has been effective in suppressing ruminal CH_4 and maintaining VFA concentration in most *in vitro* studies [11]. This essential oil directly inhibits methanogens [4]. Although the application of garlic oil *in vivo* has so far been limited, the few studies that have been conducted have not shown effective reduction in CH_4 emissions and this has been attributed to possible microbial adaptation [6] or the lower concentrations applied compared to concentrations *in vitro* [12].

Researches that specifically studied the effects of plant extracts on rumen archaea or their possible adaptation are limited [13]. The objective of this study was to determine whether archaea and other rumen microbes adapt to garlic oil, carvacrol or thymol when added at a dosage that suppresses methanogenesis in a relatively long-term *in vitro* incubation. We monitored rumen microbial populations using the quantitative real-time polymerase chain reaction (qRT-PCR) technique.

Materials and Methods

Experimental treatments and design

Experimental treatments were; garlic oil (artificial), carvacrol (98 % purity) and thymol (98 % purity) at a final concentration of 300 mg/l and a control (no additive). Garlic oil, carvacrol and thymol were dissolved in absolute ethanol to make stock solutions. Stock solutions of treatments were prepared so that 50 µl of the stock would result in a final concentration of 300 mg/l for each compound when added to 40 ml of buffered rumen fluid. In the control was added 50 µl of ethanol. Garlic oil and carvacrol were sourced from Sigma-Aldrich Company (Sigma-Aldrich, St. Louis, MO, USA) and thymol from Dae Jung Chemicals and Metals Co., LTD (Dae Jung, Siheung, Korea).

Rumen inoculum and consecutive batch culture incubation

Incubations followed the consecutive batch culture system, a method developed in the 1980s [14]. It is an *in vitro* system that allows maintaining of actively fermenting mixed populations of rumen bacteria over a long period of time. Subcultures from a previous fermentation are added into fresh buffer and incubated at the desired temperature. The process is repeated after every defined interval for as long as is necessary. In the present study we did three serial transfers at a three-day interval in 120 ml serum bottles as outlined in a study by Morgavi *et al.* [15]. Rumen inoculum was obtained from two rumen-cannulated Hanwoo (Korean native beef breed) steers. The fluid was mixed with McDougall's buffer [16] at a ratio of 1:4. Ten milliliters of the mixed rumen fluid were dispensed into serum bottles containing 30 ml of McDougall's buffer solution and 0.5 g of substrate. The substrate was made up of orchard grass and a commercial concentrate at a ratio of 60:40. The dry matter content of orchard grass and the commercial concentrate used in this study were 92.92 % and 95.46 % respectively. Organic matter, crude protein, ether extract and crude fiber content in orchard grass were 92.71, 7.77, 1.70 and 31.21 % DM whereas in the commercial concentrate they were 93.30, 13.88, 4.23 and 10.76 % DM respectively.

Parameter determination and chemical analyses

At the end of each incubation period total gas production was measured by way of displacing a glass syringe. The collected gas was stored in aluminum vacutainers for further analysis of CH_4 and H_2. The analyses of these gases were carried out using a gas chromatograph (GC-7890A, Agilent Technologies, Inc. Santa Clara, CA, USA) fitted with a thermal conductivity detector and a capillary column (Nukol™ Fused silica capillary column 30 m × 0.25 mm × 0.25 µm film thickness, Supelco, Bellefonte, USA) according to procedures outlined by Lopez *et al.* [17].

Soon after determining total gas production, a 10 ml subculture was transferred, under anaerobic conditions, into 30 ml McDougall's buffer containing respective treatments. The remaining contents were transferred into 50 ml conical tubes and pH determined using a pH meter (Mett-Toledo AG, Schwerzenbach, Switzerland). The fermentation fluid was then centrifuged at 3,100 × *g* for 20 min at 4 ˚C and 1.5 ml samples of the supernatant stored at -20 ˚C for VFA and ammonia nitrogen (N) analyses. Before analyzing for VFAs, 1 ml of each sample was pretreated with 0.2 ml of 25 % metaphosphoric acid, allowed to settle for 30 min before centrifuging at 12, 300 × *g* using a table-top centrifuge (Gyrozen mini, Seoul, Korea), then transferred into vials for analysis. VFA were analyzed using a gas chromatograph (GC-7890A, Agilent Technologies, Inc. Santa Clara, CA, USA) fitted with a flame ionizing detector and a capillary column (Carbonex™ Fused silica capillary column 30 m × 0.25 mm × 0.25 µm film thickness, Supelco, Bellefonte, USA) as outlined by Erwin *et al.* [18]. Ammonia-N was determined according to a procedure outlined by Chaney *et al.* [19].

Total deoxyribonucleic acid extraction (DNA)

The supernatant and pellet remaining in 50 ml conical tubes after sampling for VFA and ammonia-N analyses were thoroughly mixed to get a representative sample for total DNA extraction. Metagenomic DNA was extracted from 250 µl of fermentation fluid by bead beating and purified using a QIAamp® DNA stool mini kit (QIAGEN, Seoul, Korea). In order to ensure higher DNA yield, 1 ml of sample was pipetted into a micro tube using a wide end tip, centrifuged (16 000 × *g*, 4 ˚C, 5 min) and the supernatant discarded. Another 1 ml of fermentation fluid sample was added to the tube with pellet and centrifuged again (16 000 × *g*, 4 ˚C, 5 min). The supernatant was discarded and 400 µl of fermentation fluid sample added into the tube with pellet and homogenized. After allowing the mixture to settle for 2 min, 250 µl of the sample was used in the extraction of DNA. Lysis buffer was added to the sample and the mixture homogenized for 3 min using a BeadBug microtube homogenizer (Benchmark Scientific, Edison, NJ,

USA). After incubation at 70 ˚C for 15 min, with gentle shaking every 5 min, the sample was centrifuged at 4 ˚C for 5 min at 16 000 × *g*. After transferring the supernatant to a fresh Eppendorf® tube, the process was repeated after adding 300 µl of lysis buffer to the lysis tube. In order to precipitate nucleic acids, PPT mix amounting to 10 % of supernatant volume was added to each tube, inverted 4-5 times and placed on ice for 5 min. After centrifuging at 4 °C for 10 min at 16 000 × *g*, supernatants were transferred to fresh tubes and equal volumes of isopropanol added. After mixing well by pumping, tubes were left on ice for more than 30 min. Samples were then centrifuged (16 000 × *g*, 4 °C, 15 min), the supernatant discarded and 1 ml of 70 % ethanol added. Samples were centrifuged again (16 000 × *g*, 4 °C, 5 min) and then left to dry for 20 min. The remaining pellet was dissolved in 100 µl of TE buffer, and the two aliquots pooled. To remove RNA, 20 µl of DNase-free RNase (1 mg/ml) were added and incubated at 37 °C for 15 min. After this, DNA was purified using proteinase K and buffers AL, AW1, AW2 and AE of QIAGEN (QIAGEN Inc. Seoul, Korea). The DNA yield was quantified using an Epoch spectrophotometer (BioTek, Winooski, VT, USA).

Real-time PCR primers and assay conditions

A CFX96 Real-Time System (BioRad, CA, USA) was used to carry out qPCR analyses. PCR primers to amplify target 16S rRNA genes for general bacteria and archaea, and 18S rRNA gene for protozoa were chosen form literature. Primers for total bacteria used were Forward: 341f GC 5´-CCTACGGGAGGCAGCAG-3´ and Reverse: 534r GC 5´ ATTACCGCGGCTGCTGG-3´ [20]. Archaea primers were Forward: Met630f 5´-GGATTAGATACCCSGGTAGT-3´ and Reverse: Met803r 5´-GTTGARTCCAATTAAACCGC-3´ [21]. Protozoa primers were Forward: 316f 5´-GCTTTCGWTGGTAGTGTATT-3´ and Reverse: 539r 5´-CTTGCCCTCYAATCGTWCT-3´ [22].

Real-time PCR amplification for total bacteria target genes was initiated by a hot start at 94 ˚C for 5 min followed by 30 cycles of 80 ˚C for 60s, 65 °C for 60s, 55 ˚C for 60s and an extension of 72 ˚C for 3 min. As for archaea, amplification was initiated by denaturation at 94 ˚C for 4 min followed by 35 cycles of denaturation (94 ˚C for 30s), annealing (52 ˚C for 30s) and extension (72 ˚C for 60s) and then a final extension (72 ˚C for 7 min). Protozoa shared similar initial denaturation conditions with archaea, followed by 45 cycles of denaturation (94 ˚C for 30s), annealing (54 ˚C for 30s) and extension (72 ˚C for 60s) and then a final extension (72 ˚C for 6 min). Standards were created from DNA extracted from rumen fluid samples used in this study. Ten-fold series dilutions of the standards were made and used to create standard curves. The starting quantities of target genes for samples were determined from the respective standard curves of the different microbes.

Statistical analyses

To determine the overall effect of treatments, data generated were analyzed as repeated measures using MIXED procedures of SAS 9.2 [23]. The following model was used;

$$Y_{ijk} = \mu + \tau_i + \beta_j + (\tau\beta)_{ij} + \pi_k + \epsilon_{ijk}$$

Where

Y_{ijk} = Response variable
μ = overall mean common to all observations
τ_i = the ith time effect
β_j = the jth treatment effect
$(\tau\beta)_{ij}$ = interaction ith time and the jth treatment
π_k = random error with mean 0 and variance σ2, the variance between vessels within treatment
ϵ_{ijk} = random error term

Data on microbial populations were log-transformed first to achieve normality before they were subjected to the same statistical analysis as other data. The effect of treatments at each three-day interval was analyzed by one-way ANOVA. Means were separated using the LSMEANS procedure. A p-value was interpreted as significant when $p \leq 0.05$ and as tendency when $0.05 < p \leq 0.10$.

Results

Total gas, pH, methane (CH_4) and hydrogen (H_2)

The effect of treatments on rumen fermentation parameters at each sampling period (3, 6 and 9 d) are shown in Table 1. Significant overall treatment × incubation day interactions were observed on pH ($p = 0.0365$), total gas ($p = 0.0016$), CH_4 ($p = 0.0011$) and H_2 ($p = 0.0001$). Carvacrol and thymol increased ($p < 0.0001$) fermentation culture pH compared to the control. Garlic oil did not affect ($p = 0.4736$) pH. The plant-based compounds reduced ($p < 0.0001$) overall total gas compared to the control, with carvacrol and thymol suppressing ($p < 0.0001$) total gas more than garlic oil. Statistical analyses at each sampling period revealed differences in total gas between the control and garlic oil to be at day 3. Whereas garlic oil sustained a total gas output of ~ 70 ml, carvacrol and thymol reduced total gas output from 51.33 ml at day 3 to 39.67 ml at day 9. Although carvacrol and thymol lowered ($p < 0.0001$) CH_4 on the whole, the CH_4 output increased from almost 0 % to 5 % of total gas between days 3 and 9. Garlic oil on the other hand consistently lowered CH_4 to negligible amounts. Treatments influenced H_2 ($p = 0.006$). The greatest amounts of H_2 were recorded in carvacrol and thymol, followed by garlic oil, and the least was found in the control. Interesting to note are the trends in the plant-based compounds. Whereas the phenolics resulted in a diminishing H_2 output to almost zero by day 9, garlic oil conversely increased H_2 concentration.

Parameter	Incubation time (d)	Treatment[2]				P - value
		Control	Garlic oil	Carvacrol	Thymol	
pH	3	6.53 ± 0.03[b]	6.58 ± 0.01[b]	6.77 ± 0.01a	6.78 ± 0.02[a]	<0.0001
	6	6.51 ± 0.01[b]	6.48 ± 0.01[b]	6.75 ± 0.03[a]	6.71 ± 0.01[a]	<0.0001
	9	6.54 ± 0.07[b]	6.54 ± 0.02[b]	6.79 ± 0.01[a]	6.75 ± 0.01[a]	<0.0001
Total gas (ml)	3	94.33 ± 1.53[a]	69.00 ± 3.00[b]	52.00 ± 1.00[c]	51.33 ± 0.58[c]	<0.0001
	6	76.00 ± 1.00[a]	71.33 ± 3.06[a]	42.67 ± 1.15[b]	42.67 ± 2.52[b]	<0.0001
	9	84.33 ± 6.66[a]	71.67 ± 7.77[a]	39.33 ± 2.08[b]	39.67 ± 1.53[b]	<0.0001
H_2 (%)	3	0.04 ± 0.00[b]	2.12 ± 1.59[b]	12.11 ± 2.26[a]	13.40 ± 1.06[a]	<0.0001
	6	0.04 ± 0.00[c]	4.58 ± 3.85[bc]	9.80 ± 1.38[ab]	10.85 ± 1.17[a]	0.0009
	9	0.06 ± 0.01	7.68 ± 6.42	2.34 ± 1.88	4.42 ± 5.79	0.2624
CH_4 (%)	3	10.39 ± 1.44[a]	0.15 ± 0.02[b]	0.58 ± 0.41[b]	0.04 ± 0.04[b]	<0.0001
	6	8.15 ± 0.61[a]	0.45 ± 0.08[b]	1.27 ± 1.13[b]	0.66 ± 0.88[b]	<0.0001
	9	9.00 ± 1.14[a]	0.18 ± 0.05[b]	3.64 ± 0.71[b]	2.86 ± 2.45[b]	0.0004
Ammonia-N (mg/100 ml)	3	7.24 ± 0.78[a]	3.00 ± 0.41[b]	1.69 ± 0.57[bc]	1.24 ± 0.47[c]	<0.0001
	6	6.29 ± 0.85[a]	3.91 ± 0.98[b]	0.39 ± 0.29[c]	0.49 ± 0.21[c]	<0.0001
	9	7.31 ± 1.02[a]	6.65 ± 0.27[a]	1.03 ± 0.65[b]	1.06 ± 0.34[b]	<0.0001
Acetate (mM)	3	30.54 ± 6.07[a]	28.00 ± 0.48[a]	7.62 ± 0.98[b]	9.13 ± 0.62[b]	<0.0001
	6	30.24 ± 2.10[a]	28.28 ± 4.37[a]	13.27 ± 0.71[b]	15.63 ± 1.46[b]	<0.0001
	9	29.45 ± 9.72[ab]	39.09 ± 1.42[a]	16.91 ± 0.66[bc]	13.97 ± 1.14[c]	0.0009
Propionate (mM)	3	21.13 ± 1.78[a]	19.13 ± 1.02[a]	3.51 ± 0.83[b]	1.98 ± 0.47[b]	<0.0001
	6	17.52 ± 4.57[a]	19.77 ± 2.72[a]	6.67 ± 1.61[b]	3.82 ± 1.18[b]	0.0003
	9	20.04 ± 5.17[a]	23.64 ± 1.50[a]	5.36 ± 2.73[b]	1.94 ± 2.34[b]	<0.0001
nButyrate (mM)	3	7.28 ± 0.12[a]	6.46 ± 0.51[a]	3.43 ± 0.48[b]	4.39 ± 0.40[b]	<0.0001
	6	10.50 ± 1.91[a]	9.83 ± 0.45[a]	5.06 ± 0.84[b]	8.25 ± 1.08[a]	0.0022
	9	13.65 ± 1.15[a]	11.85 ± 0.52[a]	6.51 ± 0.51[b]	7.91 ± 1.48[b]	<0.0001
nValerate (mM)	3	8.91 ± 0.25[a]	5.33 ± 0.25[b]	2.40 ± 0.33[c]	1.53 ± 0.19[d]	<0.0001
	6	7.84 ± 2.49a	8.97 ± 0.74[a]	2.38 ± 0.47[b]	1.66 ± 0.36[b]	0.0003
	9	8.05 ± 3.44[a]	7.79 ± 2.08[a]	1.15 ± 0.40[b]	1.43 ± 1.60[b]	0.0055
Total VFA (mM)	3	69.85 ± 7.52a	59.83 ± 1.68[a]	19.03 ± 1.10[b]	17.63 ± 0.31[b]	<0.0001
	6	67.62 ± 6.60[a]	70.51 ± 4.21[a]	27.68 ± 2.07[b]	29.62 ± 2.64[b]	<0.0001
	9	74.52 ± 15.41[a]	83.69 ± 1.57[a]	30.15 ± 3.15[b]	26.05 ± 4.76[b]	<0.0001
A/P[1]	3	1.44 ± 0.17[b]	1.47 ± 0.08[b]	2.29 ± 0.76[b]	4.85 ± 1.48[a]	0.0032
	6	1.80 ± 0.44[b]	1.43 ± 0.03[b]	2.07 ± 0.48[b]	4.34 ± 1.36[a]	0.0058
	9	1.46 ± 0.16[b]	1.66 ± 0.16[a]	3.64 ± 1.42[a]	11.02 ± 7.17[a]	0.0389

[1]A/P: Acetate : Propionate

[2]Treatments were: control (no additive), garlic oil, carvacrol and thymol each at a final concentration of 300 mg/l;

[a,b,c,d] Significantly different ($p \leq 0.05$). Data presented as Mean ± SD; $n = 3$ per treatment

Table 1: Effect of treatments on *in vitro* rumen fermentation parameters

Ammonia nitrogen (N) and volatile fatty acids (VFAs)

The addition of carvacrol, thymol and garlic oil resulted in suppressed ($p < 0.0001$) ammonia-N, with the latter having a lesser effect compared to the phenolics. Whereas carvacrol and thymol maintained a consistent low ammonia-N concentration, ammonia-N in garlic oil steadily increased with incubation time until no difference was observed when compared to the control at day 9. Garlic oil did not alter ($p > 0.05$) individual or total VFA with the exception of lowering ($p = 0.04$) butyrate. However, carvacrol and thymol suppressed ($p < 0.0001$) individual and total VFA. Thymol resulted in a greater ($p = 0.013$) A/P ratio than the rest of the treatments.

Rumen microbial populations

The effect of treatments on microbial populations at each sampling period is shown in Table 2. No overall treatment effects ($p = 0.235$) were observed on total bacteria. On the whole, garlic oil and thymol suppressed ($p < 0.05$) methanogenic archaea compared to the control but carvacrol did not differ ($p = 0.5046$) from the control. Significant treatment × incubation day interactions ($p = 0.0116$) on methanogens were observed. Although no overall treatment effects ($p = 0.2139$) were observed on protozoa numbers, the phenolics significantly suppressed protozoa numbers compared to the control and garlic oil at day 3 of fermentation.

Rumen microbes (copy No./ ml, log_{10})	Incubation time (d)	Treatment				P - value
		Control	Garlic oil	Carvacrol	Thymol	
Total bacteria	0	9.25 ± 0.10	9.74 ± 0.37	9.46 ± 0.15	9.53 ± 0.19	0.1553
	3	10.20 ± 0.24	10.35 ± 0.34	10.09 ± 0.39	9.81 ± 0.31	0.2845
	6	10.17 ± 0.36	10.37 ± 0.54	10.39 ± 0.34	10.02 ± 0.17	0.6123
	9	10.53 ± 0.49	10.71 ± 0.19	10.36 ± 0.14	10.29 ± 0.24	0.3646
Methanogenic archaea	0	4.77 ± 0.16	4.98 ± 0.08	5.03 ± 0.18	4.92 ± 0.36	0.5314
	3	6.07 ± 0.21[a]	5.40 ± 0.48[a]	5.42 ± 0.41[a]	4.45 ± 0.13[b]	0.0026
	6	5.49 ± 0.11	4.98 ± 0.11	5.56 ± 0.53	4.72 ± 0.79	0.1796
	9	4.95 ± 0.16	4.71 ± 0.20	5.16 ± 0.04	5.18 ± 0.30	0.0632
Protozoa	0	4.90 ± 0.09	5.03 ± 0.11	4.84 ± 0.04	4.85 ± 0.09	0.0809
	3	4.06 ± 0.15	4.07 ± 0.10	3.66 ± 0.17[b]	3.46 ± 0.15[b]	0.0020
	6	3.33 ± 0.28	3.92 ± 0.36	3.97 ± 0.50	3.96 ± 0.06	0.1279
	9	2.51 ± 0.38	1.95 ± 1.69	1.58 ± 1.38	2.00 ± 0.49	0.7957

[a,b] Significantly different ($p \leq 0.05$). Data presented as Mean ± SD; $n = 3$ per treatment; Treatments were: control (no additive), garlic oil, carvacrol and thymol each at a final concentration of 300 mg/l

Table 2: Effect of treatments on microbial counts (copy No./ ml of fermentation fluid, log_{10}) after 3, 6 and 9 days of *in vitro* fermentation

Discussion

The observed increase in pH at the inclusion of carvacrol and thymol is in agreement with earlier studies [3,4,24]. This indicates a negative effect on overall fermentation, a hypothesis supported by suppressed VFA concentrations observed in this study. The suppressed gas production and CH_4 in thymol and carvacrol is in agreement with previous studies [4,25]. Garlic oil has also been reported to suppress gas production [26], which is apparent at day 3, but the difference between garlic oil and the control group diminished in subsequent samplings. Observations of garlic oil selectively inhibiting methanogens directly as supported by the accumulation of H_2 are consistent with earlier studies [5,26]. The increase in CH_4 over time in carvacrol and thymol, combined with a decline in H_2 is in response to the multiplication of methanogens. As methanogens increased they used up more H_2 even though overall fermentation remained low. This corroborates the proposition by Waghorn *et al.* [27] that plant compounds can reduce CH_4 by suppressing fiber digestion. Thymol, however, as evidenced by lower methanogen numbers, is either more potent than carvacrol at the same concentration or has a broader antimicrobial activity. Carvacrol and thymol also exhibited defaunation qualities in early fermentation, a factor that could have aided in the decrease in CH_4 as methanogens are associated with protozoa [28-30]. The suppression of CH_4 without affecting overall rumen fermentation by garlic oil indicates selective direct inhibition of methanogens [31]. The observed decline in methanogen abundance in the control group could be associated with a decline in protozoa and the low pH of the culture contents [32]. Also, slight oxygen (O_2) contamination during subculture transfers cannot be completely ruled out although measures were taken to maintain an anaerobic environment. Methanogens are highly sensitive to even low O_2 concentration exposure [33]. The low protozoa concentrations even in the control group can be explained by observations made by Hungate [34] that it is not easy to achieve protozoa numbers *in vitro* as those observed in the rumen because of problems in maintaining pH and also waste removal as in the rumen. Although no specific bacteria species were identified as the ones adapting to carvacrol or thymol in this study, microbial adaptation is the most plausible explanation to the present observations. In a study of the effect of a combination of essential oils on pure cultures of rumen microbes, McIntosh *et al.* [35] reported that *Prevotell bryantii* and *Prevotell ruminocola* adapted to essential oils and managed to grow in higher doses.

Previous researchers have consistently reported a decrease in molar proportions of individual and total VFA concentration at the addition of carvacrol or thymol [4,24,36]. The low VFA concentrations are strongly related to the negative influence of carvacrol and thymol on dry matter degradability as reported by Martinez *et al.* [36]. Carvacrol and thymol possess antibacterial properties and have been found to be effective against both Gram-positive and Gram-negative bacteria [37,38]. Their mode of action involves increasing cell membrane fluidity, leakage of plasma protons and potassium ions, a collapse of the cell membrane, cessation of ATP synthesis and ultimately cell death [39]. Just as was observed in this study, Evans *et al.* [3] also observed an increase in the A/P ratio in thymol. Contrary to reports by Martinez *et al.* [36] who used an essential oil from *Thymus hyemalis* rich in carvacrol, carvacrol in this study did not affect the A/P ratio.

Essential oils obtained from thyme and oregano together with garlic oil reduced ammonia-N *in vitro [*25]. Cardozo *et al.* [40] also reported a decrease in ammonia-N at the addition of garlic oil. These reductions in ammonia-N at the inclusion of these plant-based compounds indicate a suppression of the deamination process. Research has reported on phenolics and garlic oil inhibiting the major rumen bacteria involved in deamination [41,42]. Busquet *et al.* [7], however, found no significant effect of carvacrol on ammonia-N. The discrepancy could be attributed to the low concentration of carvacrol (2.2 mg/l) applied in their study.

Conclusion

Results obtained in this study suggest that rumen microbes may adapt to carvacrol and thymol but not to garlic oil. The phenolic compounds suppressed overall fermentation, hence low VFA concentrations and CH_4 production. However, the antimethanogenic effects of carvacrol and thymol diminished over time, although it could not be determined whether the adaptation would be partial or complete. Whereas garlic oil and thymol suppressed methanogens, carvacrol did not differ from the control suggesting that thymol is more potent at the same concentration or has a broader antibacterial activity than carvacrol. Garlic oil selectively inhibited methanogens. Carvacrol and thymol exhibited defaunation qualities after 3 days of fermentation, but these diminished over time.

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