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EFFECTS OF RUMEN EXPOSURE TO ANISE OIL ON RUMINAL FERMENTATION AND BIOHYDROGENATION OF *N* – 3 POLYUNSATURATED FATTY ACIDS

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ABSTRACT

This study evaluated the effect of four weeks of microbial exposure to anise oil (ANO) on the fermentation activities of ruminal micro-organisms and the biohydrogenation of *n*-3 polyunsaturated fatty acids (PUFA). A total of six Hartline × Texel cross lambs were used. Three of the lambs were randomly assigned to the basal diet (basal diet group, BDG) and the remaining three lambs were offered basal diet with ANO (anise oil group, AOG) for four weeks. The rumen fluid donor lambs were offered water and hay *ad libitum* and supplemented with additional 400 g/sheep/day of concentrate plus 2.4 g/sheep/day of ANO (for the AOG). The ANO was thoroughly mixed with the concentrate prior to feeding. The total amount (400 g) of lamb finisher cubes offered per sheep/day was divided into two equal parts (200 g) and fed at 08.00 and 16.00 hours, respectively. After the four weeks' exposure period, lambs were slaughtered and ruminal fluid (RF) was collected from each of the lambs on BDG and on AOG and used in a 48 h *in vitro* batch culture system to study the fermentation of a 70: 30 grass hay (*Lolium perenne)* and concentrate (lamb finisher) diet. The study was a 2 (batches of rumen fluid: BDG and AOG) × 2 (doses of ANO: 0 and 200 mg/L) factorial design experiment. Results showed that total volatile fatty acid (TVFA) in RF extracted from lambs on the BDG (242.0 mM) was similar to that in the AOG (242.2 mM). Concentrations of TVFA in *in vitro* digests were similar between groups on the BDG (68.6 mM) and on the AOG (66.4 mM), but differed between levels of anise oil (0 versus 200 mg/L). The levels of NH_3-N were not different (P>0.05) between the RF collected from AOG and BDG. However, *in vitro* results showed that AOG 200 mg/L induced a 20% decrease on the concentration of NH_3-N in fermentation vessels, relative to BDG 0 mg/L. The *in vitro* digesta incubated in RF from the AOG (i.e. AOG 200 mg/L) maintained higher concentrations of PUFA (C18:2 *n*-6 and *n*-3 PUFA), *trans*vacceric acid and lower concentration of stearic acid. This suggests that there was no rumen adaptation within the period of trial. This study concludes that anise oil is potentially a useful feed additive to optimise the fatty acid composition of ruminant feedstuffs, if these effects are repeated in meat and milk.

KEYWORDS: Rumen filtrate, anise oil, biohydrogenation, polyunsaturated fatty acid

INTRODUCTION

Essential oils (EOs) which are also known as volatile oils are aromatic (relating to the smell and taste) oily liquids (Guenther, 1948; Burt, 2004), extracted from different parts of plants such as the stem, leaves, seeds, roots, etc. The presence of these EOs in plants are thought to be responsible for the unique smell produced by plants due to the different smells and aroma produced by different EOs (Szumacher-Strabel and Cieslak, 2012). These complex compounds are synthesized as secondary metabolites from aromatic plants, particularly, spices and herbal plants (Bakkali *et al*., 2008; Patra, 2011).

In our previous studies (Eburu and Chikunya, 2015a; b), it was established that certain EOCs (e.g. anethole and 4-allylanisole) and whole EOs (e.g. anise oil) when added at 200 mg/L can significantly suppress biohydrogenation of PUFA without detrimental effects to fermentation and VFA concentrations. However, it is not clear whether these effects are sustained in the medium and long term. Available evidence from continuous culture studies and long term *in vivo* trials suggest that the benefits associated with the use of essential oil as feed additives may decline due to two possibilities: (i) adaptation of individual microbial species to EOs or, (ii) shifts in microbial populations following long-term use of essential oil (Gladine *et al*., 2007). In addition, only few studies have been conducted *in vivo* to investigate the possibility of microbial adaptation to essential oils in the rumen as a measure of their potential effectiveness as feed additives. Previous studies with the use of specific blend of essential oil (BEO) compounds consisting mainly of limonene, guaiacol and thymol observed that ruminal N- metabolism was modified by supplementing with BEO (McIntosh *et al*., 2003; Molero *et al*., 2004; Newbold *et al*., 2004). About four weeks' period of microbial adaptation was suggested as the minimum time required to modify Nitrogen metabolism in the rumen by some of these studies (Wallace *et al*., 2003;

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Molero *et al*., 2004; Castillejos *et al.,* 2007). However, to date, there is no similar report about the adaptation period of microbes to EOs and their corresponding effects on fatty acid metabolism in the rumen. The aim of the present study was to examine effects of four weeks' period of microbial adaptation to anise oil on metabolism of PUFA and biohydrogenation end-product. Anise oil (200 mg/L) was identified as the most effective substance to both prevent PUFA disappearance and maintain optimal VFA concentration in our previous experiment (Eburu and Chikunya, 2015). The active compounds of Anise oil (*Pimpinella anisum*) are *trans* – anethol (82.7%), carryophyllene (3.8%) and limonene (2.3%). This study therefore evaluated the effect of four weeks of microbial adaptation to anise oil (ANO) on the fermentation activities of ruminal micro-organisms and the biohydrogenation of *n*-3 polyunsaturated fatty acids (PUFA).

MATERIALS AND METHODS

Animal management and collection of rumen fluid/sampling

In this study, six Hartline × Texel cross lambs (mean weight 42.0 ±2.5kg) were used as rumen fluid donors. The rumen fluid donor lambs were offered water and hay *ad libitum* and supplemented with additional 400 g/sheep/day of concentrate. The total amount (400 g) of lamb finisher cubes offered per sheep/day was divided into two equal parts (200 g) and fed at 08.00 hours and 16.00 hours. Three lambs were randomly assigned to the basal diet (basal diet group, BDG) and the remaining three lambs were fed basal diet with ANO (2.4 g/sheep/day ANO, AOG) for four weeks. The 2.4 g/day/sheep of anise oil was thoroughly mixed with the lamb finisher cubes prior to feeding. The dose (2.4 g/sheep/day) was estimated based on an estimated rumen volume of about 10 litres (as rumen volume equals body weight) (Owens and Goetsch, 1988) and a dilution rate of about 100 mg/h. The total amount of fluid estimated to flow through the rumen per 24 h/day was 24 litres which equals about 100 mg/L (a dose which was observed to maintain the level of TVFA similar to the control in the previous experiment) (Eburu and Chikunya, 2015). Feed was withdrawn from the ewes at 18.00 hours on the eve of the day of slaughter. Sheep were taken to the abattoir (Humphreys and Sons, Chelmsford-Essex) at 07.00 hours in the morning and were slaughtered at 07.30 hours. Whole rumens were then collected and immediately sealed in tough plastic bags to prevent oxygen entry and transported in insulated boxes to maintain rumen temperature to the Lordship Science laboratory. The rumens were incised with a scalpel blade and rumen contents were scooped and the liquor strained through 2 layers of cheesecloth. After straining, the remaining solids were mixed with a volume of buffer equal to the rumen liquor removed, and homogenized using a kitchen blender for about 45 seconds to detach rumen microbes attached to solids (modified from Theodorou *et al*., 1994). The mixture was re-strained with 2 layers of cheesecloth and the filtrate added to the rumen fluid to constitute the buffer rumen fluid mixture as the final inoculum. The mixed fluid was held in a water bath maintained at 39ºC and was flushed with CO_2 to expel oxygen before being dispensed into the *in vitro* incubation flasks.

Basal feedstock, treatments and *in vitro* incubation

The basal feedstock was mixtures of good quality rye-grass hay (*Lolium perenne*), lamb finisher concentrate, whole ground linseed and fish oil. The basal feedstock used in this study was made from the mixture of a 70:30 rye- grass (*Lolium perenne*, Patterns Farm, Chelmsford, UK) and lamb finisher concentrate (W & H Marriage & Sons LTD, Chelmsford, Essex), respectively. The ingredients and chemical composition of the basal feedstock used in incubation are shown in Table 1. A 70: 30 mixture of the grass hay and concentrate respectively was formulated and milled through 1 mm screen (Glen Creston Ltd, Stammore, England). This diet was supplemented with 32.5 g oil/kg from a mixture on an oil basis, of 60% of fish oil (Sigma-Aldrich Co. Ltd., UK) and 40% of ground linseed (NBTY Europe LTD, Burton-Upon-Trent), as extra sources of *n*-3 PUFA. Linseed was used as a major source of C18:3 *n*-3 and fish oil was included as the main source of C20:5 *n* -3 and C22:6 *n* -3. In order to make 1 kg of the basal feedstock used in incubations, 700 g of hay, 250 g of concentrate, 30 g of ground linseed and 20 g of fish oil were mixed together as shown in Table 1. The composition of the concentrate used according to the supplier (W & H Marriage & Sons LTD, Chelmsford, Essex) was a mixture of wheat (19.6%), wheat feed (40.1%), molasses (3%), palm kernel extract (12%), sunflower extract (5%), limestone flour (2%), salt (0.8%), mixer oil (0.5%), millspec molasses (6%), spray oil (0.5%), ammonium chloride (0.3%) and malt nuts (10%).

The effect of anise oil was evaluated using the *in vitro* gas production batch culture method described by Theodorou *et al*. (1994). Anise oil was purchased from Sigma-Aldrich Co. Ltd., UK (Table 2) and was stored at the required temperatures specified on delivery notes prior to use. The effect of four weeks adaptation of rumen microbes to ANO was examined *in vitro*, using rumen fluid extracted from both basal diet group (BDG) and anise oil group (AOG) in a 2 (batches of rumen fluid: BDG and AOG) × 2 (doses of ANO: 0 and 200 mg/L) factorial design experiment with six replicates as follows: (1) fluid from BDG plus 0 mg/l of anise (BDG 0 mg/l); (2) fluid from BDG plus a single dose of 200 mg/l of anise oil (BDG 200 mg/l); (3) fluid from AOG plus 0 mg/l of anise oil (AOG 0 mg/l); and (4) fluid from AOG plus a single dose of 200 mg/l of anise (AOG 200 mg/l). In total 100 serum bottles were incubated, each bottle contained 1 g of feed substrate, 200 or 0 mg/l of anise oil, 80 ml anaerobic buffer and 20 ml inoculum and the bottle sealed with rubber cork before incubation.

Sample collection and preservation

Gas pressure in the bottles during incubation was measured from all the replicates at various times (3, 6, 9, 12, 24, 36 and 48 h) using a pressure transducer (Bailey and Mackey Ltd., Birmingham, UK) which was connected to a digital read-out voltmeter. The pressure was read on the transducer and then the gas was released to return the head-space gas pressure to zero. The bottles were agitated by shaking before returning to the incubator. Fermentation was stopped (at 12, 24 and 48 h) by freezing the contents of incubation bottles at -20ºC for 5 minutes. After 5 minutes, serum bottles were brought to room temperature, and then 3 replicates of each treatment were taken to determine ammonia (5 ml) and volatile fatty acids (VFA, 4 ml). The aliquots for

ammonia were preserved by mixing 5 ml sample with 5 ml of 1M HCL. Volatile fatty acid samples (4 ml) were mixed with 1 ml of a deproteinising solution and frozen (-20ºC) until chemical analysis.

Table 1: The ingredients, chemical composition and fatty acid content of the basal feedstock used in incubations

Components	Composition
Feed ingredient (g/kg fresh)	
Hay	700.0
[1]Concentrate	250.0
Linseed	30.0
Fish oil	20.0
Chemical composition of basal feedstock (g/kg DM)	
Dry matter	921.0
Crude protein	123.6
Neutral detergent fibre	405.2
Acid detergent fibre	219.5
Ether extract	54.3
Fatty acid composition (g/100 g TFA)	
Linolenic (C18:3 n-3)	21.9
Linoleic (C18:2 n-6)	12.9
Palmitic (C16:0)	12.4
Oleic (C18:1 n-9)	10.0
Eicosapentaenoic (C20:5 n-3)	4.9
Myristic (C14:0)	4.3
Palmitoleic (C16:1)	4.0
Docosahexaenoic (C22:6 n-3)	3.7
Stearic (C18:0)	2.5
Vaccenic (C18:1)	1.6
Arachidonic (C20:4 n-6)	0.1
Remaining fatty acids	21.6
Total fatty acids (mg/g)	59.1

[1]**Concentrate=** W & H Marriage & Sons LTD, Chelmsford, Essex

Table 2: Description of the essential oils used in this experiment as purchased from Sigma-Aldrich

Name of EO	Abbreviation	Catalogue number	Type	Density
Anise oil	ANO	10521	Anise seed oil, natural identical	0.980 g/ml

Supplier: Sigma-Aldrich Co. Ltd., UK; FCC = food chemicals codex; FG = food grade

Chemical analysis

The concentration of NH_3-N in digesta was analysed using the phenol-hypochlorite method (Weatherburn, 1967; Broderick and Kang, 1980) adopted for use on the plate reader. The concentration of volatile fatty acid (VFA) was determined by Gas chromatography (GC) as described by Ottenstein and Bartley (1971).

The concentration of fatty acids in feed and freeze dried samples were extracted by direct saponification method described by Enser *et al*. (1998).

Experimental design and statistical analysis

The study (RCBD) was a 2 (batches of rumen fluid: BDG and AOG) $\times$ 2 (doses of ANO: 0 and 200 mg/L) factorial design experiment. Therefore, data were analysed by TWO-WAY analysis of variance (ANOVA) with batches

of rumen fluid and dose of anise as the main effects using GenStat 16th edition. Differences between treatments were declared by least significance difference (LSD) and significance was declared at P< 0.05. Data were analysed separately for each time point (12, 24 and 48 h).

RESULTS

Fermentation parameters

The effects of four weeks of microbial adaptation to anise oil on animal performance and concentrations of total volatile fatty acids (TVFA, mM), molar proportion of individual VFA (mM/mol TVFA) and NH_3-N (mM) *in vivo* are shown in Table 3. The dry matter intake was significantly higher in lamb that received anise oil compared to the control. Mean values were 18.5 and 18.9 kg/week (SEM= 0.05, P<0.05, Table 3), for the control and the anise oil group of lambs, respectively. The initial and final body weight gain were not different (P=0.05) between the two groups of lambs (Table 3). The concentrations of NH_3-N, TVFA and molar proportions of individual VFA were not affected by treatments. However, the acetate to propionate ratio was lower in the AOG than the BDG. Mean values were 3.4 and 3.1 (SEM= 0.008, P=0.05, Table 3), for the BDG and the AOG, respectively. The effects of treatments on cumulative gas production (ml/g OM), NH_3-N (mM), TVFA (mM) and molar proportion of individual VFA (mM/mol TVFA) *in vitro* are presented in Table 4. In this experiment the fluid exposed to anise oil (AOG) and the unexposed fluid (BDG) maintained similar levels of total gas production.

The molar proportion of acetate was significantly higher (P<0.001) in cultures with the AOG than the BDG. Mean values (average of doses within a fluid type) were 24 h (48 h in brackets) 61.3 (61.1) and 63.2 (62.6) mM/mol TVFA for the BDG and AOG, respectively. Although there was no observed difference between doses of anise on the proportion of acetate at 24 h, levels were different at 48 h, such that the 200 mg/L of both BDG and AOG maintained higher levels of acetate than the 0 mg/L (Table 4).

The proportion of propionate was significantly lower (P<0.001) in cultures with the AOG compared to the BDG. Mean values (mean of doses within a fluid type) for 24 h were 20.0 and 18.1 and for 48 h were 20.2 and 18.7 mM/mol TVFA (Table 4), for the BDG and AOG, respectively. The proportion of propionate was not affected by the dose of anise (irrespective of fluid type) at 24 h, but levels were different at 48 h, with the 200 mg/L causing the higher reduction in propionate compared to the lower dose (0 mg/L). The molar proportion of butyrate was not affected by treatment except at 24 h where the AOG reduced (P<0.001) it compared to the BDG (mean values 8.5 and 8.0 mM/mol TVFA for the BDG and AOG, respectively). The acetate to propionate ratios were maintained at higher levels in the AOG relative to the BDG at both 24 and 48 h. Treatment effects caused minor changes to the concentrations of branched chain volatile fatty acids (BCVFA) such as isovaleric, valeric and isobutyric acids (Table 4).

Table 3: Effect of four weeks of rumen adaptation to anise oil on animal performance and concentrations of total volatile fatty acids (mM), molar proportion of individual VFA (mM/mol TVFA) and NH_3-N (mM) *in vivo*

	Treatments			
	Basal diet group	**Anise oil group**	**SEM**	**LOS**
Animal performance				
Intake (kg/week)	18.50	18.90	0.05	<0.001
Initial body weight (kg)	42.01	42.02	0.01	NS
Final body weight (kg)	44.02	44.03	0.01	NS
Volatile fatty acid concentration				
TVFA	242.00	242.2	18.6	NS
Acetate	68.30	65.40	2.57	NS
Propionate	20.00	21.6	2.49	NS
Butyrate	7.70	8.40	0.47	NS
Isobutyric	0.88	0.88	0.00	NS
Isovaleric	0.67	0.73	0.00	NS
Valeric	0.56	0.53	0.00	NS
A/P ratio	3.40	3.10	0.008	=0.05
Ammonia concentration				
Ammonia	3.2	2.6	0.61	NS

NS= Not significant at P<0.05. Note: Because of the small sample size (n=3) for the parameters measured in this table, differences between mean was calculated using the t-Test.

Table 4: Effect of four weeks of rumen adaptation to anise oil on total gas (ml/g OM), ammonia concentration (mM), total VFA (mM) and molar proportion of individual VFA (mM/mol TVFA) in cultures during 48 h *in vitro* incubation

		Treatments									
		Basal diet group		Anise oil group		S.E.D			Significance		
Parameter	Time	0 mg/l	200	0 mg/l	200	Fluid	Dose	F × D	Fluid	Dose	F × D
Gas	12	96.2	87.2	96.0	90.4	3.08	3.08	4.36	NS	=0.028	NS
	24	133.2	121.6	131.5	123.7	2.93	2.93	4.14	NS	=0.004	NS
	48	164.8	144.3	159.4	145.4	2.88	2.88	4.08	NS	<0.001	NS
NH_3-N	12	4.0	3.9	4.0	3.6	0.33	0.33	0.47	NS	NS	NS
	24	5.0	4.7	5.3	4.0	0.29	0.29	0.41	NS	=0.017	NS
	48	6.0	5.9	6.0	4.8	0.33	0.33	0.47	NS	NS	NS
TVFA	24	70.2	67.0	67.3	65.5	1.69	1.9	2.39	NS	NS	NS
	48	81.5	74.6	80.0	75.0	2.10	2.10	2.97	NS	<0.001	NS
Acetate	24	60.9	61.6	63.0	63.4	0.41	0.41	0.58	<0.001	NS	NS
	48	60.1	62.1	62.0	63.2	0.37	0.37	0.52	<0.001	<0.001	NS
Propionate	24	20.4	19.6	18.4	17.7	0.44	0.44	0.62	<0.001	NS	NS
	48	21.7	18.6	20.0	17.3	0.39	0.39	0.55	<0.001	<0.001	NS
Butyrate	24	8.6	8.4	7.9	8.0	0.19	0.19	0.27	=0.014	NS	NS
	48	8.0	7.7	7.9	7.9	0.16	0.16	0.23	NS	NS	NS
Isobutyric	24	0.87	0.83	0.87	0.82	0.013	0.013	0.019	NS	=0.003	NS
	48	0.96	0.96	0.98	0.94	0.023	0.023	0.032	NS	NS	NS
Isovaleric	24	1.12	1.07	0.98	0.88	0.027	0.027	0.038	<0.001	=0.011	NS
	48	1.31	1.43	1.21	1.04	0.049	0.049	0.070	<0.001	NS	=0.006
Valeric	24	0.98	0.97	1.00	0.99	0.011	0.011	0.015	NS	NS	NS
	48	1.05	1.19	1.19	1.31	0.022	0.022	0.031	<0.001	<0.001	NS
A/P ratio	24	3.0	3.1	3.4	3.6	0.09	0.09	0.13	<0.001	NS	NS
	48	2.8	3.3	3.1	3.7	0.09	0.09	0.12	<0.001	<0.001	NS

S.E.D= Standard error of difference; F × D = fluid by dose interaction, NS= Not significant at P<0.05

Effect of EOC on fatty acid metabolism

The effects of treatments on the concentration of selected fatty acids (g/100 g TFA) are presented in Table 5. The levels of C14:0 were not affected by treatments. There was a significant interaction which indicates that addition of 0 and 200 mg/L of anise to the fluid exposed to anise oil (i.e. AOG 0 mg/L and AOG 200 mg/L) significantly increased the concentration of C16:1 compared with the respective 0 and 200 mg/L of the unexposed fluid (BDG) at 12 h. Mean values were 3.5, 3.6, 3.8 and 3.8 g/100 g TFA (sed= 0.03, P<0.016, Table 5), for BDG 0, BDG 200, AOG 0 and AOG 200, respectively.

The concentrations of C18:0 were reduced (P<0.001) in cultures with the AOG relative to the BDG (Table 5). Mean values were 14.2 and 11.2 g/100 g TFA (12 h, sed= 0.15, P<0.001), 15.5 and 11.9 g/100 g TFA (24 h, sed= 0.21, P<0.001) and for 48 h were 16.8 and 12.9 g/100 g TFA (sed= 0.45, P<0.001), for BDG and AOG, respectively. The 200 mg/L regardless of the type of fluid (BDG or AOG) maintained higher levels of C18:0 than the 0 mg/L of all fluids. The concentrations of C18:1 *n*-9 in cultures were not different (P<0.05) between the BDG and AOG (Table 5). At the dose level, the 200 mg/L of all fluid type maintained higher levels of C18:1 *n*-9 than the 0 mg/L. Mean values were 7.9, 8.0, 7.7 and 8.2 (12 h); 6.3, 7.3, 6.1 and 7.8 (24 h) and for 48 h were 4.5, 5.6, 3.1 and 5.4 g/100 g TFA for BDG 0, BDG 200, AOG 0 and AOG 200, respectively.

The concentrations of C18:1 *trans* 11 at 12 and 24 h were significantly higher in cultures with the AOG than the BDG. Mean values were 3.6 and 3.8 (12 h, sed= 0.02, P<0.001, Table 5) and 24 h were 2.9 and 3.4 g/100 g TFA (sed=0.05, P<0.001, Table 5), for the BDG and AOG, respectively. There was no difference in the dose of anise oil at all time points except at 48 h where the AOG 200 mg/L had higher level of 18:1 *trans* 11 than the rest of the treatments. Average values were 1.4, 1.4, 1.4 and 1.6 g/100 g TFA BDG 0, BDG 200, AOG 0 and AOG 200, respectively. The concentrations of C18:2 *cis*-9 *trans* 11CLA in cultures with BDG and AOG were similar (Table 5). The addition of different doses of anise oil (0 and 200 mg/L) had no effect on the levels of C18:2 *trans* 11CLA at all time of incubation except at 12 h, where the levels of C18:2 *trans* 11CLA in cultures were maintained in a linear pattern (dose-response). Mean values were 0.25, 0.26, 0.27 and 0.28 g/100 g TFA BDG 0, BDG 200, AOG 0 and AOG 200, respectively.

At both 12 and 24 h period, the unexposed fluid (BDG) and exposed fluid (AOG) maintained a similar level of C18:2 *n*-6 and C18:3 *n*-3 (Table 5). In contrast, the concentrations of C18:2 *n*-6 and C18:3 *n*-3 at 48 h were higher in vessels with AOG relative to BDG. Mean

values were 5.1 and 6.8 (C18:2 *n*-6, sed=0.44, P<0.049) and for C18:3 *n*-3 were 5.7 and 7.5 g/100 g TFA (sed=0.36, P<0.022), for BDG and AOG, respectively. The 200 mg/L of all fluids maintained higher levels of C18:2 *n*-6 and C18:3 *n*-3 compared to the control. There was a significant interaction at 24 h time point, which showed that, the concentrations of C18:2 *n*-6 and C18:3 *n*-3 in vessels where AOG 200 was added were higher than their corresponding values in BDG 200 (Table 5). Mean values were 2.7, 4.1, 2.6 and 4.9 g/100 g TFA (18:2 *n*-6, sed= 0.29, P<0.035) and for C18:3 *n*-3 were 3.4, 5.7, 3.3 and 7.0 g/100 g TFA (sed= 0.45, P<0.031), for BDG 0, BDG 200, AOG 0 and AOG 200, respectively.

At 12 h the concentration of C20:4 *n*-6 was higher (P<0.025) in cultures with the AOG compared to the BDG (Table 5). By contrast, the levels of C20:4 *n*-6 at 24 and 48 h were lower in vessels with AOG than BDG. There was significant interaction at 12 h period of incubation, which showed that supplementing cultures with AOG 200 mg/L contained higher concentration of ∑MUFA compared with the addition of BDG 200 mg/L.

The vessel contents of ∑PUFA were significantly higher in the AOG than the BDG at 12 h. Mean values were 41.3 and 42.5 g/100 g TFA (sed= 0.17, P<0.002, Table 6), for BDG and AOG, respectively. The addition of AOG 200 mg at 24 h had the highest content of ∑PUFA compared to the control. Mean values were 12.8, 15.0, 11.1 and 18.1 g/100 g TFA (sed= 1.43, P<0.025, Table 6), for BDG 0, BDG 200, AOG 0 and AOG 200 mg/L, respectively. At 48 h the levels of TFA in AOG exceeded (P<0.001) the concentration in BDG (mean values were 64.4 and 75.9 mg/g for BDG and AOG respectively). Total fatty acid content (mg/g) in all fluids was not affected by the dose of anise.

The ratio of *n*-6/*n*-3 in cultures at all time points (12, 24 and 48 h) was significantly higher in the BDG than the AOG (Table 6). The vessel content of *n*-6/*n*-3 in cultures was higher in BDG 0 mg/L compared to the AOG 0 mg/L and this was true for BDG 200 mg/L relative to AOG 200 mg/L. Average concentrations were 0.46, 0.44, 0.44 and 0.44 (12 h, sed=0.003, P<0.001); 0.45, 0.46, 0.41 and 0.44 (24 h, sed=0.007, P<0.032), and for 48 h were 0.50, 0.46, 0.44 and 0.47 (sed=0.013, P<0.001), for BDG 0, BDG 200, AOG 0 and AOG 200 mg/L, respectively.

The biohydrogenation of C18:2 *n*-6 and C18:3 *n*-3 in vessels with the higher dose of anise (200 mg/L) were maintained at lower levels compared with the levels of biohydrogenation in cultures with BDG 0 mg/L. There were some significant interactions at both 12 and 24 h such that the biohydrogenation of C18:2 *n*-6 and C18:3 *n*-3 increased significantly in response to treatment in the sequence: values for C18:2 *n*-6 were BDG 0 mg/L (50.5 and 74.1) > AOG 0 mg/L (48.0 and 73.3) > BDG 200 mg/L (36.9 and 60.4) > AOG 200 mg/L (30.1 and 48.3 g/100 g), for 12 (sed=1.26, P<0.05) and 24 h (sed=2.79, P<0.010, Table 7), respectively. Similar values for C18:3 *n*-3 were BDG 0 mg/L (60.9 and 80.5) > AOG 0 mg/L (58.4 and 80.4) > BDG 200 mg/L (44.8 and 68.0) > AOG 200 mg/L (37.8 and 56.2 g/100 g), for 12 (sed=1.37, P<0.032) and 24 h (sed=2.51, P<0.004, Table 7), respectively.

At 12 and 24 h, the mean biohydrogenation (mean across all doses within a fluid type) of 20:5 *n*-3 and 22:6 *n*-3 were lower in AOG relative to levels in BDG (Table 7). The biohydrogenation of 20:5 *n*-3 and 22:6 *n*-3 decreased as the dose of anise oil increased in fermentation vessels, such that the highest dose (200 mg/L) of all fluids maintained lower levels of disappearance compared with the control.

Table 5: Effects of four weeks of rumen adaptation to anise oil on concentration of selected fatty acids (g/100 g total fatty acids) in cultures during 48 h *in vitro* incubation

		Treatments				S.E.D			Significance		
		Basal diet group		Anise oil group							
Fatty acid	Time	0 mg/l	200	0 mg/l	200	Fluid	Dose	F × D	Fluid	Dose	F × D
C14:0	12	5.0	5.0	5.0	5.0	0.01	0.01	0.11	NS	NS	NS
	24	5.0	5.0	5.0	5.0	0.10	0.10	0.10	NS	NS	NS
	48	5.0	5.0	5.0	5.0	0.10	0.10	0.10	NS	NS	NS
C16:0	12	15.0	14.0	15.0	14.0	0.10	0.10	0.10	NS	<0.001	NS
	24	15.0	15.0	15.0	15.0	0.20	0.20	0.30	NS	NS	NS
	48	17.0	15.0	16.0	15.0	0.30	0.30	0.40	NS	=0.004	NS
C16:1	12	3.6	3.6	3.8	3.8	0.02	0.02	0.03	<0.001	NS	=0.016
	24	3.3	3.3	3.5	3.6	0.07	0.07	0.10	0.003	NS	NS
	48	3.0	3.2	3.2	3.3	0.09	0.09	0.13	NS	NS	NS
C18:0	12	6.9	7.3	5.3	5.9	0.15	0.15	0.21	<0.001	=0.002	NS
	24	7.4	8.1	5.3	6.6	0.21	0.21	0.30	<0.001	<0.001	NS
	48	8.0	8.8	5.8	7.1	0.45	0.45	0.64	<0.001	=0.029	NS
C18:1 *n*-9	12	7.9	8.0	7.7	8.2	0.07	0.07	0.09	NS	<0.001	NS
	24	6.3	7.3	6.1	7.8	0.32	0.32	0.45	NS	<0.001	NS
	48	4.5	5.6	3.1	5.4	0.74	0.74	1.04	NS	=0.029	NS
C18:1 trans 11	12	1.8	1.8	1.9	1.9	0.02	0.01	0.02	<0.001	NS	NS
	24	1.4	1.5	1.7	1.7	0.05	0.05	0.07	<0.001	NS	NS
	48	1.4	1.4	1.4	1.6	0.06	0.06	0.08	NS	=0.033	NS
18:2 tr 11CLA	12	0.25	0.28	0.26	0.27	0.008	0.008	0.011	NS	=0.034	NS
	24	0.17	0.18	0.17	0.17	0.013	0.013	0.019	NS	NS	NS
	48	0.15	0.16	0.16	0.18	0.016	0.016	0.023	NS	NS	NS
C18:2 *n*-6	12	5.1	6.5	5.1	6.6	0.06	0.06	0.08	NS	<0.001	NS
	24	2.7	4.1	2.6	4.9	0.20	0.20	0.29	NS	<0.001	=0.035
	48	2.3	2.8	2.3	4.0	0.44	0.44	0.63	=0.049	=0.006	NS
C18:3 *n*-3	12	6.8	9.7	7.0	9.9	0.10	0.10	0.15	NS	<0.001	NS
	24	3.4	5.7	3.3	7.0	0.32	0.32	0.45	NS	<0.001	=0.031
	48	2.1	3.6	3.0	4.5	0.36	0.36	0.50	=0.022	<0.001	NS
C20:4 *n*-6	12	0.5	0.5	0.6	0.5	0.01	0.01	0.01	=0.025	NS	NS
	24	0.6	0.6	0.5	0.5	0.02	0.02	0.02	=0.030	NS	NS
	48	0.6	0.6	0.5	0.5	0.01	0.01	0.01	<0.001	NS	NS
C20:5 *n*-3	12	3.1	3.5	3.3	3.7	0.03	0.03	0.03	<0.001	<0.001	NS
	24	1.6	2.4	2.3	3.1	0.15	0.15	0.21	<0.001	<0.001	NS
	48	1.2	1.6	1.2	2.2	0.27	0.27	0.38	NS	=0.016	NS
C22:6 *n*-3	12	2.4	2.6	2.6	2.7	0.03	0.03	0.04	<0.001	<0.001	NS
	24	1.8	2.2	2.2	2.4	0.07	0.07	0.10	<0.001	<0.001	NS
	48	1.6	1.7	1.8	2.1	0.13	0.13	0.18	NS	NS	=0.049

S.E.D= Standard error of difference; F × D = fluid by dose interaction, NS= Not significant at P<0.05

Table 6: Assessing the effects of rumen adaptation to anise oil on the concentration of sum of fatty acids (g/100 g TFA) and content of total fatty acids (mg/g) on animals treated with Basal or Anise oil diets for the four weeks prior to slaughter. Rumen Fluid was subsequently incubated *in vitro* for 48 hours, (reading taken at 12, 24 and 48 hours).

		Treatments				S.E.D			Significance		
		Basal diet group		Anise oil group							
Fatty acid	Time	0 mg/l	200	0 mg/l	200	Fluid	Dose	F × D	Fluid	Dose	F × D
	12	32.4	31.0	32.1	29.8	0.20	0.20	0.28	<0.001	<0.001	=0.039
RFA	24	36.2	33.9	38.1	32.2	0.97	0.97	1.37	NS	<0.001	NS
	48	37.6	34.5	42.4	37.9	1.24	1.24	1.75	=0.003	=0.006	NS
	12	26.7	26.2	25.6	25.2	0.18	0.18	0.25	<0.001	=0.020	NS
∑SFA	24	28.1	28.7	26.0	26.8	0.24	0.24	0.35	<0.001	=0.011	NS
	48	29.7	29.3	27.1	27.8	0.64	0.64	0.90	=0.005	NS	NS
	12	13.3	13.3	13.4	13.9	0.06	0.06	0.08	<0.001	<0.001	=0.006
∑MUFA	24	11.0	12.1	11.3	13.1	0.37	0.37	0.52	NS	<0.001	NS
	48	8.9	10.3	7.7	10.3	0.85	0.85	1.20	NS	=0.031	NS
	12	18.2	23.1	18.8	23.7	0.17	0.17	0.24	=0.002	<0.001	NS
∑PUFA	24	12.8	15.0	11.1	18.1	1.01	1.01	1.43	NS	<0.001	=0.025
	48	7.4	10.5	8.9	14.8	1.58	1.58	2.24	NS	=0.010	NS
	12	34.7	36.8	37.1	35.5	0.79	0.79	1.11	NS	NS	=0.029
TFA	24	35.1	33.9	35.9	36.5	0.93	0.93	1.32	NS	NS	NS
	48	33.8	30.6	39.2	36.7	1.48	1.48	2.10	<0.001	NS	NS
	12	0.46	0.44	0.44	0.44	0.002	0.002	0.003	<0.001	=0.002	<0.001
***n*-6/*n*-3**	24	0.45	0.46	0.41	0.44	0.005	0.005	0.007	<0.001	=0.002	=0.032
	48	0.50	0.46	0.44	0.47	0.009	0.009	0.013	=0.028	NS	<0.001
	12	0.68	0.88	0.73	0.94	0.010	0.010	0.015	<0.001	<0.001	NS
P/S	24	0.46	0.52	0.43	0.68	0.035	0.035	0.050	=0.092	<0.001	=0.017
	48	0.27	0.38	0.30	0.53	0.071	0.071	0.100	NS	=0.028	NS

S.E.D= Standard error of difference; F × D = fluid by dose interaction, RFA= remaining fatty acids, ∑SFA= sum of saturated fatty acids, ∑MUFA= sum of monounsaturated fatty acids, ∑PUFA= sum of polyunsaturated fatty acids, TFA= total fatty acids, P/S= ∑PUFA/∑SFA, *n*-6/*n*-3= sum of *n*-6 divided by sum of *n*-3 fatty acids, NS= Not significant at P<0.05

Table 7: Assessing the effects of rumen adaptation to anise oil on biohydrogenation of PUFA (g/100 g TFA) when animals were treated with basal or Anise oil diets for four weeks prior to slaughter. Rumen fluid was subsequently incubated *in vitro* for 48 hours, (reading were taken at 12, 24 and 48 hours).

		Treatments				S.E.D			Significance		
		Basal diet group		Anise oil group							
Fatty acid	Time	0 mg/l	200	0 mg/l	200	Fluid	Dose	F × D	Fluid	Dose	F × D
	12	50.5	36.9	48.0	30.1	0.89	0.89	1.26	<0.001	<0.001	=0.053
C18:2 *n*-6	24	74.1	60.4	73.3	48.3	1.97	1.97	2.79	=0.004	<0.001	=0.010
	48	82.8	71.0	77.4	61.4	4.51	4.51	6.38	NS	=0.006	NS
	12	60.9	44.8	58.4	37.8	0.97	0.97	1.37	<0.001	<0.001	=0.032
C18:3 *n*-3	24	80.5	68.0	80.4	56.2	1.78	1.78	2.51	=0.003	<0.001	=0.004
	48	87.7	77.0	83.1	74.4	2.18	2.18	3.08	NS	<0.001	NS
	12	31.8	23.0	24.7	11.5	0.90	0.90	1.28	<0.001	<0.001	NS
C20:5 *n*-3	24	65.8	48.4	48.7	25.7	3.22	3.22	4.56	<0.001	<0.001	NS
	48	73.7	61.1	73.2	53.0	6.00	6.00	8.48	NS	=0.013	NS
	12	19.7	13.2	7.9	3.9	1.46	1.46	2.06	<0.001	=0.002	NS
C22:6 *n*-3	24	41.2	27.6	22.8	11.4	2.68	2.68	3.79	<0.001	<0.001	NS
	48	45.5	31.1	36.1	35.3	4.15	4.15	5.87	NS	NS	NS

S.E.D= Standard error of difference; F × D = fluid by dose interaction, NS= Not significant at P<0.05

DISCUSSION

Dry matter intake was marginally higher (by 2%) in sheep that received anise oil (AOG) compared to the control (BDG). However, final body weight gains were not affected by treatments (BDG and AOG). Nevertheless, it could be speculated that the AOG that had higher feed intake but without higher weight gain may have excreted more nutrients than the BDG. By contrast, previous *in vivo* studies where higher doses: 500 mg/day of cinnamaldehyde (Busquet *et al*., 2003; Calsamiglia *et al*., 2007), and a mixture of eugenol (90 mg/day) plus 180 mg/day of cinnamaldehyde (Cardozo *et al*., 2006) were used observed negative effect on feed intake. The observed difference in feed intake between the present study and some previous studies (Busquet *et al*., 2003; Cardozo *et al*., 2006; Calsamiglia *et al*.,

2007) could be due to the different doses or type of EOs/EOCs or the diet used.

Fermentation parameters

It was observed that although the BDG and AOG maintained similar levels of total gas production, BDG 200 and AOG 200 caused 7.9% (average) inhibition of total gas relative to the control. This level of reduction of total gas in cultures supplemented with 200 mg/L from both sources of rumen fluid (AOG and BDG) in the current study is similar and consistent with the level of inhibition of gas production when 200 mg/L of anise oil was used in the previous study (Unpublished). A number of studies have indicated that different blend of essential oil decreased proteolysis and amino acid deamination (McIntosh *et al*., 2003; Molero *et al*., 2004; Newbold *et al*., 2004; Castillejos *et al*., 2007). Majority of these studies proposed that about four weeks period of microbial adaptation to blend of essential oil was the minimum period of time necessary to observe the effects of mixtures of essential oil on protein metabolism in the rumen (Wallace *et al*., 2003; Molero *et al*., 2004; Castillejos *et al*., 2007). In the current *in vivo* study, the concentrations of NH_3-N from the two fluids (BDG and AOG) were not affected by treatments. This observation is in agreement with Newbold et al. (2004) who reported that 110 mg/d of blend of EO (Crina® ruminants; Akzo Surface Chemistry Ltd., Herfordshire, UK) did not affect the concentration of NH_3-N measured *in vitro*. Furthermore, Castillejos *et al.* (2007) observed that the concentration of NH_3-N was not affected by supplementing 110 mg/L of a blend of essential oil (Crina® Ruminant; AKZO NOBEL/CRINA S.A., Gland, Switzerland). The constituents of the Crina® Ruminant were guaiacol, thymol and limonene. However, both studies (Newbold *et al*., 2004; Castillejos *et al.,* 2007) reported that 24% and 14% respectively, of ammonia N was decreased when measured *in vitro* from rumen fluid collected from sheep adapted with blend of essential oil for four weeks. In the current *in vitro* study, we observed that the addition of AOG 200 mg/L but not of BDG 200 mg/L induced a 16% reduction on NH_3-N concentration after 24 h. This observation suggests reduced deamination or decreased concentration of HAP (although not tested). Some studies have demonstrated that some HAP bacteria such as *Peptostreptococcus anaerobius* and *Clostridium sticklandii* were more sensitive than others such as *Clostridium aminophilum* to the Crina blend of essential oil (McIntosh *et al*., 2003). Contrary to the results obtained in the current study, previous results (Eburu and Chikunya, 2015a; b) indicate that anise oil (300 and 200 mg/L, respectively), did not change the levels of NH_3-N in rumen contents. These results indicate the possibility that the accumulated residual effects of feeding anise oil to lambs (for four weeks) resulted to the decreased concentration of ammonia, suggesting that ruminal microbes need adaptation time of about four weeks in order to change the levels of NH_3-N in the rumen.

After four weeks period of microbial adaptation to anise oil, TVFA concentrations in the rumen (*in vivo*) and *in vitro* were not different between the BDG and AOG, which implies that anise oil, at the dose tested in this study, neither affect feed intake nor alter the utilization of energy. These results are consistent with results of other previous studies (Wallace *et al*., 2002; Newbold *et al*., 2004). In addition, Castillejos *et al*. (2007) observed in a dual flow continuous culture studies that supplementing 650 mg/day of a blend of essential oil (Crina® Ruminant; AKZO NOBEL/CRINA S.A., Gland, Switzerland) did not alter the concentration of VFA (*in vitro* and *in vivo*). Since the absorption of VFA in the rumen is proportional to its ruminal concentration (Lopez *et al*., 2003), this study suggests that absorption of VFA would also not be affected with the inclusion of 2.4 g/sheep/day of anise oil. Although the molar proportions of individual VFA were not affected by treatment *in vivo*, results were different *in vitro*. The unadapted fluid plus 200 mg/L of anise oil (BDG 200 mg/L) did not affect the molar proportions of acetate, propionate and the acetate to propionate ratio at 24 h. In contrast, AOG 200 mg/L increased acetate and reduced propionate. These results between *in vitro* and *in vivo* studies are in agreement with the observation of Castillejos *et al*. (2007) who reported that although blend of essential oil did not affect the concentrations of total and individual VFA *in vivo*, levels were affected when rumen fluid collected from sheep adapted to blend of essential oil was used in *in vitro* trial. The concentrations of branched-chain volatile fatty acids (BCVFA) such as isobutyric and isovaleric in AOG 200 mg/L were reduced which agreed with the reduced concentration of NH_3-N in those cultures. These results are consistent with decreased deamination process (Allison *et al*., 1962).

Effect of EOC on fatty acid metabolism

Saturated fatty acids such as C14:0 and C16:0 have the capacity to increase the plasma cholesterol levels by suppressing and saturating low-density lipoprotein (LDL) receptors (Keys *et al*., 1957). In the current study, the levels of C14:0 were not affected by treatments, but the g/100 g concentration of C16:0 was considerably reduced with the addition of 200 mg/L of anise oil (irrespective of fluid) relative to the control. This agrees with the previous reports (Eburu and Chikunya, 2014, 2015a; b) and suggests that anise oil could potentially reduce the levels of plasma cholesterol through decreasing the concentration of palmitic (C16:0) acids. The average concentration of C18:0 was reduced in cultures supplemented with AOG relative to the BDG. The 200 mg/L regardless of the type of fluid (AOG or BDG) maintained higher levels of C18:0 than the 0 mg/L. At 12 and 24 h, the concentration of C18:1 *trans* 11 (vaccenic acid) was significantly higher in cultures with the AOG relative to the BDG. These results suggest that anise oil modified the biohydrogenation of fatty acids by reducing the production of stearic acid (the end-product of biohydrogenation) and maintained higher levels of C18:1 *trans* 11 than the control cultures. Reduction in the content of C18:0 and higher levels of C18:1 *trans* 11 suggests that supplementing anise oil might reduce the activity of *Butyrivibrio proteoclasticus*, the ruminal microorganism responsible for converting C18:1 *trans* 11 to stearic acid during the reductase-step of biohydrogenation (Moon *et al*., 2008). These results agree with the reports from our preliminary *in vitro* studies (Eburu and Chikunya, 2014; 2015a; b). In those studies, both anise oil and its main active constituent compound (anethole), maintained high concentration of

PUFA in cultures as a consequence of decreased biohydrogenation.

The concentrations of C18:2 *cis*-9 *trans* 11CLA in cultures at 12 h were maintained in a linear dose-response pattern. This is consistent with reports from our previous study where anise oil maintained higher levels of C18:2 *trans* 11 CLA (Eburu and Chikunya 2015a, b). This intermediate of biohydrogenation (C18:2 *cis*-9 *trans* 11 CLA) is formed in the first step during the transformation of *cis*-9, *cis*-12 18:2 by the linoleic acid isomerase (LA-I) (Jenkins *et al*., 2008; Kim *et al*., 2009; Buccioni *et al*., 2012). Results of the current study suggest that anise oil at the level tested facilitated the activities of the linoleic acid isomerase which regulates the formation of *cis*-9 *trans* 11 CLA from *cis*-9, *cis*-12 18:2.

The content of C18:2 *n*-6 and C18:3 *n*-3 rapidly decreased with time and the biohydrogenation of C18:2 *n*-6 and C18:3 *n*-3 was similar to previous *in vitro* (Beam *et al*., 2000; Sinclair *et al*., 2005) and *in vivo* (Wachira *et al*., 2000; Scollan *et al*., 2001) studies. After 48 h time point, the concentrations of C18:2 *n*-6 and C18:3 *n*-3 were higher in vessels with AOG than in BDG, and the 200 mg/L of all fluids maintained higher levels of C18:2 *n*-6 and C18:3 *n*-3 compared to the control. At 24 h time point, the concentrations of C18:2 *n*-6 and C18:3 *n*-3 in vessels with added AOG 200 mg/L were higher than their corresponding values in BDG 200 mg/L. This significant interaction proposes the possibility of the residual effects of feeding anise oil to lambs (for four weeks) on the maintenance of the concentration of PUFA by decreasing biohydrogenation. The biohydrogenation of these C-18 PUFAs was similar to their concentrations with AOG 200 mg/L indicating the highest potency to decrease the disappearance of PUFA. These results are consistent with those of Vasta *et al*. (2013) who observed that after 3 months of supplementing the diet of Barbarine lambs with 400 ppm of Artemisia essential oil, the concentrations of C18:1 *trans* 11, C18:2 *cis*-9 *trans* 11, C18:2 *n*-6, C18:3 *n*-3 and MUFA in the muscle were increased compared to the control. Furthermore, other studies reported that the fat content of milk from dairy cows was increased by feeding 5 g/day/cow and 2/day/cow of alium and juniper essential oils, respectively (Yang *et al*., 2007). By contrast, Chaves *et al*. (2008) reported that supplementing garlic, juniper berry and cinnamaldehyde at 200 mg/kg of dry matter to growing lambs did not modify the fatty acid profile of back and liver fat. Other studies observed that monoterpenes blend consisting of β-pinene, linalool, α-pinene, *p*-cymene at 0.43 g/kg of dry matter intake did not change the fatty acid profile of milk (Malecky *et al*., 2009). In another study, supplementing the diet of dairy cows with 1 g/day of cinnalmaldehyde did not affect the fatty acid composition of milk (Benchaar *et al*., 2007b). The difference between the present study and previous studies (Chaves *et al*., 2008; Malecky *et al*., 2009) for composition of fatty acid could be due to the difference in the chemical structure of essential oils and the duration of exposure. These factors (the chemical structure of essential oils and the duration of exposure) could affect the applicability of *in vitro* results and the actual response from animal performance *in vivo* due to possible microbial adaptation. This is why other researchers suggest that sufficient time should be allowed in *in vitro* trials in order to study the possibility of such adaptations (Calsamiglia *et al*., 2007).

The extent of disappearance of C20:5 *n*-3 and C22:6 *n*-3 in vessels increased as the time of incubation progressed. After 24 h, the quantity of the content of 20:5 *n*-3 in vessel which disappeared was highest in the control (73.7%) and supports previous reports from *in vivo* studies where the biohydrogenation of C20:5 *n*-3 and C22:6 *n*-3 from fish oil range from 72 to 93 g/100 g TFA (Wachira *et al*., 2000; Scollan *et al*., 2001; Chikunya *et al*., 2004). The disappearance of C22:6 *n*-3 which was less than the biohydrogenation of C20:5 *n*-3 was also consistent with other previous *in vitro* reports where the biohydrogenation of C22:6 *n*-3 was less than 50 g/100 g (Ashes *et al*., 1992; Sinclair *et al*., 2005). Rumen fluid from sheep adapted with anise oil (AOG) maintained higher levels of C20:5 *n*-3 and C22:6 *n*-3 and reduced their biohydrogenation than BDG, and all treatments maintained a dose-related response effect on the levels of C20:5 *n*-3 and C22:6 *n*-3. As the concentrations of these fatty acids reflect the biohydrogenation data, it indicates that disappearance of these PUFA was substantially reduced in the AOG. However, there was no significant interaction between fluid and dose of anise on the concentration and biohydrogenation of these long chain PUFAs.

The addition of graded doses of anise (0 and 200 mg/L) sequentially increased the concentrations of ∑PUFA and P/S ratio and progressively decreased the concentrations of ∑*n*-6/∑*n*-3 and ∑SFA, relative to the control. These increased concentrations of ∑PUFA, P/S ratio and progressive decrease on the concentrations of ∑*n*-6/∑*n*-3 and ∑SFA with 200 mg/L of anise oil were higher in vessels with the AOG 200 mg/L relative to the BDG 200 mg/L. This interaction implies that feeding 2.4 g/day/sheep of anise oil for 4 weeks improved the concentrations of ∑PUFA, P/S ratio, ∑*n*-6/∑*n*-3 and ∑SFA than just the supplementation of cultures with 200 mg/L of anise oil.

This microbial adaptation study did not measure the stability of anise oil (the used essential oil) in the rumen. However, this assessment would have been achieved by measuring the amount of the oil in the diet and examination of the feaces for the same purpose. There is increasing evidence that essential oils and their constituent compounds are anaerobically biodegraded to a number of other compounds. This suggests that adaptation may result from the biotransformation of the active form of a compound to a less active form. In anaerobic environment, facultative bacteria such as *Alcaligenes defragrans* degrade monoterpenes such as limonene into 2-carene or α-terpinene depending on the absence or availability of nitrate, respectively (Heyen and Harder, 1998). Other microbes such as *E.coli* produce carveol, perillyl alcohol, perillic acid and carvone from the biotransformation of monoterpenes such as limonene (Cheong and Oriel, 2000; Mars *et al*., 2001).

In summary, as an indication of modified fatty acid profile in the present study, the fluid from lambs adapted

with 2.4 g/sheep/day for four weeks contained higher levels of individual and total PUFA and reduced concentration of C18:0 and ∑SFA than the unadapted fluid. This study indicates that there was no microbial adaptation to anise oil (2.4 g/sheep/day) during this experimental period (four weeks), and suggests that at the dose tested, anise oil sustain microbial inhibition and prevented any form of microbial adaptation process.

CONCLUSION

In this four weeks' adaptation study, fluid from lambs exposed to anise oil maintained higher concentrations of total and individual PUFA, *trans* vaccenic acid and lower concentration of stearic acid. These results indicate that there was no microbial adaptation to anise oil in the rumen during the four weeks' period of study. These results suggest that anise oil is potentially a useful feed additive to optimise the fatty acid composition of ruminant food products, if these effects are observed in meat and milk.

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