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IN VITRO EFFECTS OF ORGANIC ACID AND PLANT OILS ON SHEEP RUMEN FATTY ACID COMPOSITION

Z. Váradyová, K. Mihaliková, T. Laho, S. Kišidayová and D. Jalč

Institute of Animal Physiology, Slovak Academy of Sciences, Šoltésovej 4-6, 040 01 Košice - Slovak Republic
Corresponding author E-mail address: varadyz@saske.sk

ABSTRACT

The effects of diets (i.e., high fibre, HF and high concentrate, HC) supplemented with either fumaric acid (FUM; 8 mmol/L) or maleic acid (MAL; 8 mmol/L) and different plant oils (35.0 g/kg dry matter; sunflower, SO; rapeseed, RO; and linseed, LO) on rumen sheep fatty acids (FA) concentration and biohydrogenation (BH) in batch cultures were examined. The oils rich in linoleic acid (SO, 535 g/kg of FA), oleic acid (RO, 539 g/kg of FA) and α-linolenic acid (LO, 538 g/kg of FA) were examined. The diets and organic acids with oils (AO) affected the concentration of almost all FA in batch cultures. Compared with the control, higher concentrations of trans-vaccenic acid occurred for the HF-diet with FUM+SO and MAL+SO ($P<0.001$) and for HC-diet with FUM+SO, MAL+SO, FUM+RO and MAL+RO ($P<0.01$ and $P<0.001$). The BH of oleic acid in both the HF- and HC-diets with organic acids and oils was decreased when compared with the control. The combination of FUM and MAL, especially with SO and RO, might positively enhance the concentration of FA and some FA intermediates and decreases the BH of C_{18} FA in rumen fluid in batch cultures.

Key words: batch cultures, fumaric acid, maleic acid, sunflower oil, rapeseed oil, linseed oil, sheep rumen.

INTRODUCTION

The current world trend in ruminant nutrition has increased the demand for feed additives to ruminant diets that alter the microbial ecosystem in order to improve the efficiency of converting feed and to produce consumable products for humans. Unsaturated fatty acids (UFA) released during the hydrolysis of dietary lipids by ruminal microbes are subjected to the process of biohydrogenation (BH), which requires H_2 (Jin *et al.*, 2008). The major intermediates of the BH of polyunsaturated fatty acids (PUFA) by rumen bacteria are conjugated linoleic acids (cis9, trans11 $C_{18:2}$; CLA) and trans-vaccenic acid (trans11 $C_{18:1}$; TVA). Since the removal of CLA as an intermediate depends on its BH, it may be possible to disrupt this process by providing alternative electron acceptors. Organic acids act as an alternative to antimicrobial compounds by stimulating rather than inhibiting specific ruminal microbial populations and their activity (Martin, 1998). The beneficial effect of organic acids on rumen fermentation is similar to effect of the ionophore monensin and their addition to ruminant feeds affects rumen fermentation, causing a shift from the production of methane to the production of propionate (Callaway and Martin, 1996). Fumarate, as a propionate precursor, also acts as an H_2 acceptor, accepting one pair of electrons during its conversion into propionate (Martin and Streeter, 1995; López *et al.*, 1999). It is also known that the incubation of organic acids with linoleic acid affects rumen fermentation and competes with methane and the BH of linoleic acid in the utilization of metabolic H_2 by rumen microbes (Li *et al.*, 2009; 2010; Liu *et al.*, 2008). We previously examined the effect of plant oils and organic acids on rumen fermentation parameters in vitro as well as the effect of plant oils on fatty acid (FA) profiles of rumen fluid of sheep (Jalč *et al.*, 2002; Váradyová *et al.*, 2007). However, only limited information has been reported on the combination effects of both additives (i.e., organic acids and plant oils) on rumen lipid metabolism (Li *et al.*, 2011). The present experiment was focused on examining the additive effect of organic acids (i.e., maleic and fumaric acid) and plant oils (sunflower, rapeseed, and linseed) as feed supplements in batch cultures fermentation incubated with high fibre and a high concentrate diet.

MATERIALS AND METHODS

Animals and sampling: Rumen inoculum was obtained from three rumen-cannulated Slovak Merino sheep (aged 4 years, mean body weight 44 ± 2.8 kg) fed 960 g dry matter (DM) of meadow hay and 240 g DM of crushed barley grain in two equal meals per day. The sheep were housed separately in pens and had free access to water. Rumen contents were collected three hours after the morning feeding using a manual vacuum pump into a pre-warmed (39 ± 0.5°C) collection vessel (2 L) filled with CO_2. Within 15 minutes, the rumen contents from all sheep were combined proportionally and blended under CO_2 in a pre-warmed blender for 30 s, squeezed through four layers of cheesecloth into a pre-warmed flask under a constant stream of CO_2, and kept in a water

bath at 39 ± 0.5°C prior to adding into the fermentation flasks.

In vitro incubation: The rumen contents was mixed (1:1) with McDougall's buffer (McDougall, 1948) containing (g/L): $NaHCO_3$ 9.24, $Na_2HPO_4 .12H_2O$ 7.12, NaCl 0.47, $MgCl_2$ 0.47, KCl 0.45 and $CaCl_2$ 0.055; under continuous flushing with CO_2. After mixing, 35 mL of rumen content inocula was pumped by an automatic pump into the preheated fermentation bottles (120 mL serum bottles) containing diet substrates. The fermentation bottles were then filled up with CO_2 and closed with butyl rubber stoppers and aluminum screw caps. The incubation was performed in the incubator for 72 h at 39 ± 0.5°C with occasional gentle shaking.

Substrates and additives: Meadow hay and barley grain were used as the components (substrates) of a high fibre diet (HF, 800:200 w/w) and high concentrate diet (HC, 500:500 w/w), respectively. The substrates were ground and sieved through a 0.15-0.4 mm screen, bulked, and then stored in sealed plastic containers. The ground substrates were added (0.20 g MH and 0.05 g BG) into each individual batch culture fermentation bottle (100 mL) for the HF-diet. Similarly, the ground substrates were added (0.125 g MH and 0.125 g of BG) into each individual batch culture fermentation bottle (100 mL) for the HC-diet. This was followed by the addition of the plant oils – sunflower (SO), rapeseed (RO) or linseed (LO) – in doses of 35.0 g/kg DM to each of the fermentation bottles, respectively. The oils were obtained from commercial sources. Finally, fumaric acid (FUM, Sigma-Aldrich Co., St. Louis, MO, USA) and maleic acid (MAL, Fluka Chemika, Steinheim, Switzerland) were added in doses of 8 mmol/L. The nutrient and fatty acid composition of the MH and BG substrates and the plant oils is presented in Table 1. Six replicate fermentation bottles of the HF-diet containing substrates, additives and inoculum (HF+FUM, HF+MAL, HF+FUM+SO, HF+FUM+RO, HF+FUM+LO, HF+MAL+SO, HF+MAL+RO, HF+MAL+LO) were used for each experimental group. Six replicate fermentation bottles of the HC-diet containing substrates, additives and inoculum (HC+FUM, HC+MAL, HC+FUM+SO, HC+FUM+RO, HC+FUM+LO, HC+MAL+SO, HC+MAL+RO, HC+MAL+LO) were used for each experimental group. An additional six bottles with the HF- or HC- diets were used as the controls (containing substrates and inoculum, but no additives).

Fatty acid analysis: After 72 h of fermentation, the contents of the fermentation bottles were freeze-dried using a ThermoSavant Micromodulyo freeze-drier (Thermo Savant MicroModulyo, NY, USA), placed in pre-cleaned high density polyethylene flasks, and kept in the dark at laboratory temperature until analyzed. The temperature of freeze-drying of fermentation bottles was -50°C. Lipids were extracted from 0.5 g of freeze-dried, 72 h-fermented inocula using a 2:1 mixture of chloroform: methanol, with samples purified using 20% HCl (Bligh and Dyer, 1959). The fatty acid analysis was determined as described by Váradyová *et al*., (2008) on Perkin-Elmer Clarus 500 gas chromatograph (Perkin-Elmer, Inc. Shelton, CN, USA) equipped with a DB-23 capillary column (60 m × 0.25 mm, film thickness 0.25 µm, Agilent Technologies, Inc., Santa Clara, CA, USA) and a flame ionization detector (constant flow, hydrogen 40 mL/min, air 400 mL, 260°C).

Calculations and statistical analysis: Biohydrogenation (BH) of the FA ($C_{18:1}$, $C_{18:2,}$ $C_{18:3n-3}$) was calculated as described by Fievez *et al.*, (2007). Statistical analysis used two-way analysis of variance (Graphpad Instat, Graphpad Software Inc., San Diego, CA, USA) as a 2 × 2 × 3 factorial design that represented two diet groups (HF and HC), two organic acid groups (FUM, MAL) and three groups of plant oils with organic acids (SO+FUM, RO+FUM, LO+FUM, SO+MAL, RO+MAL and LO+MAL, respectively). Effects included in the model were diets (D), organic acids (A), organic acids with oils (AO) and interaction between D × AO. Differences from control were analyzed using a Bonferroni post-test and considered to be significant when $P<0.05$. Values are presented in the tables are means ± standard error of means (SEM).

RESULTS

With the exception of myristic acid and CLA, the diets (D) affected the concentration of all FA in batch cultures ($P<0.05$ and $P<0.01$; Table 2). The organic acids (A) were effective in the concentration of myristic acid ($C_{14:0}$), stearic acid ($C_{18:0}$), oleic acid ($C_{18:1}$) and linoleic acid ($C_{18:2}$) ($P<0.05$ and $P<0.01$). The organic acids with oils (AO) affected the concentration of all FA ($P<0.05$ and $P<0.001$). In both the HF- and HC-diets with FUM+RO and MAL+RO, the contents of myristic ($C_{14:0}$) and oleic acid ($C_{18:1}$) were higher compared with the control ($P<0.05$, $P<0.01$ and $P<0.001$). The TVA content was higher with the HF-diet with FUM+SO and MAL+SO ($P<0.001$) and with the HC-diet with FUM+SO, MAL+SO, FUM+RO and MAL+RO ($P<0.01$ and $P<0.001$) when compared with the control. The interaction of the diets and organic acids with oils (D × AO) in the concentration of palmitic acid ($C_{16:0}$), stearic acid ($C_{18:0}$), linoleic acid ($C_{18:2}$) and α-linolenic acid ($C_{18:3}$) ($P<0.05$, $P<0.01$ and $P<0.001$) were detected. The BH of all FA were influenced by diets ($P<0.05$ and $P<0.01$) and organic acids with oils ($P<0.05$ and $P<0.001$; Table 3). In addition, the BH of oleic acid ($C_{18:1}$) was affected by organic acids ($P<0.01$). Compared with the control, the BH of oleic acid ($C_{18:1}$) in both the HF- and HC-diets with both organic acids (FUM, MAL) and all

Table 1. Nutrient and fatty acid composition of diet substrates and plant oils (sunflower oil, SO; rapeseed oil, RO and linseed oil, LO).

	MH	BG	SO	RO	LO
Dry matter (g/kg)	924	900	-	-	-
Nutrient composition (g/kg of DM)					
Nitrogen	8.90	22.1	-	-	-
Crude protein	53.3	120	-	-	-
NDF	576	261	-	-	-
ADF	368	67.4	-	-	-
Ash	80.0	37.0	-	-	-
IVDMD	580	893	-	-	-
Hemicellulose	208	194	-	-	-
Cellulose	292	53.8	-	-	-
Fatty acid composition (g/kg of FA)					
$C_{14:0}$ myristic	21	12	1.0	0.5	0.8
$C_{16:0}$ palmitic	330	288	57	47	53
$C_{16:1}$ palmitoleic	21	11	1.4	2.2	1.2
$C_{18:0}$ stearic	48	27	32	36	37
$C_{18:1}$ oleic	101	204	329	539	196
$C_{18:2}$ linoleic	183	364	535	205	152
$C_{18:3}$ α-linolenic	138	32	10	94	538
Saturated FA	400	330	97	89	95
Monounsaturated FA	145	224	333	558	200
Polyunsaturated FA	352	402	545	306	697

MH: meadow hay; BG: barley grain; SO: sunflower oil; RO: rapeseed oil; LO: linseed oil; FA: fatty acids; DM: dry matter; NDF: neutral detergent fibre; ADF: acid detergent fibre; IVDMD: in vitro dry matter degradability

Table 2. Composition of fatty acids (g/kg of FA) in batch cultures with high fibre (HF, 800:200 w/w) and high concentrate (HC, 500:500 w/w) diets, organic acids (fumaric acid, FUM; maleic acid, MAL) and plant oils (sunflower oil, SO; rapeseed oil, RO; linseed oil, LO).

Diets	Acids	Oils	$C_{14:0}$	$C_{16:0}$	$C_{18:0}$	$C_{18:1}$	TVA	$C_{18:2}$	CLA	$C_{18:3}$
High fibre	Control		39.1	333	328	42.2	32.6	64.2	20.1	19.7
	FUM	None	34.2	302	339	36.3	33.6	79.9	25.4	18.6
		SO	34.2	350	334	36.1	71.1^{c}	117	29.9	17.5
		RO	48.3^{b}	368	383	60.2^{c}	40.8	87.4	32.2	19.5
		LO	43.6	378	398	42.4	42.9	86.1	27.4	20.7
	MAL	None	36.0	333	364	38.0	38.5	85.2	22.1	22.2
		SO	30.3	202	355	35.9	79.3^{c}	98.7	26.0	18.2
		RO	44.6^{b}	338	320	63.8^{c}	41.6	89.5	37.3	16.1
		LO	41.9	369	397	40.7	41.7	88.4	23.5	19.8
High concentrate	Control		38.3	347	312	48.0	39.1	63.7	21.9	14.6
	FUM	None	36.0	389	334	38.9	31.2	68.4	25.8	14.4
		SO	32.1	229	297	42.6	73.0^{c}	123	28.2	16.9
		RO	47.9^{b}	360	217	70.3^{c}	52.3^{b}	89.1	39.0	24.7
		LO	42.6	354	351	42.9	43.8	86.9	26.2	32.5
	MAL	None	30.1	396	375	42.2	36.6	68.4	21.3	19.5
		SO	34.2	247	235	44.7	76.6^{c}	128	27.5	20.2
		RO	47.7^{b}	393	227	61.1^{c}	53.4^{b}	85.1	39.0	24.3
		LO	42.7	346	365	43.6	43.7	82.7	22.5	30.3
SEM			1.87	26.9	18.6	2.26	2.77	4.14	5.78	1.88
Significance: Diets (D)			NS	*	**	**	*	*	NS	**
Acids (A)			*	NS	*	**	NS	*	NS	NS
Acids and Oils (AO)			***	***	***	***	***	***	*	***
D × AO			NS	*	***	NS	NS	**	NS	***

$C_{14:0}$: myristic acid; $C_{16:0}$: palmitic acid; $C_{18:0}$: stearic acid; $C_{18:1}$: oleic acid; TVA: trans-vaccenic acid; $C_{18:2}$: linoleic acid; CLA: cis9, trans11 $C_{18:2}$ conjugated linoleic acid; $C_{18:3}$: α-linolenic acid; SEM: standard error of means; Control containing substrate, but no supplements.
*P<0.05; **P<0.01; ***P<0.001; NS, not significant. [a]P<0.05; [b]P<0.01; [c]P<0.001 differences from respective controls.

Table 3. Biohydrogenation of fatty acids (%) in batch cultures containing high fibre (HF, 800:200 w/w) and high concentrate (HC, 500:500 w/w) diets with organic acids (fumaric acid, FUM; maleic acid, MAL) and plant oils (sunflower oil, SO; rapeseed oil, RO and linseed oil, LO).

Diets	Acids	Oils	$C_{18:1}$	$C_{18:2}$	$C_{18:3}$
High fibre	Control		40.5	87.7	90.9
	FUM	None	46.1	77.1	88.9
		SO	32.7[a]	78.5	85.6
		RO	31.5[a]	73.4	81.8
		LO	31.3[a]	67.2	80.9
	MAL	None	46.6	80.8	78.6
		SO	30.7[a]	79.6	80.4
		RO	31.4[a]	72.9	81.3
		LO	34.0[a]	68.4	80.9
High concentrate	Control		44.6	83.9	96.8
	FUM	None	56.8	81.9	97.3
		SO	28.3[c]	85.6	88.2
		RO	32.7[b]	66.7	84.1
		LO	33.5[a]	74.8	87.6
	MAL	None	56.8	83.1	98.4
		SO	27.2[c]	76.0	86.7
		RO	33.2[c]	77.8	85.7
		LO	33.1[b]	68.3	77.5
SEM			2.75	3.18	3.95
Significance: Diets (D)			**	**	*
Acids (A)			**	NS	NS
Acids and Oils (AO)			***	***	*
D × AO			NS	**	NS

$C_{18:1}$: oleic acid; $C_{18:2}$: linoleic acid; $C_{18:3}$: α-linolenic acid; SEM: standard error of means; Control containing substrate, but no supplements.
*P<0.05; **P<0.01; ***P<0.001; NS, not significant. [a]P<0.05; [b]P<0.01; [c]P<0.001 differences from respective controls.

oils (SO, RO, LO) was lower (P<0.05, P<0.01 and P<0.001). The effect of the diets (P<0.01), organic acids with oils (P<0.001) and the interaction of the diets and organic acids with oils (D × AO) in the BH of linoleic acid ($C_{18:2}$) were detected.

DISCUSSION

We observed the effect of experimental diets on the concentration of almost all FA in batch cultures. However, reports on the effects of high fibre and high concentrate diets and increased proportions of concentrate in the diets on FA concentration are inconsistent. Some authors reported that increasing the amount of concentrate in the diets may also result in a further increase in the amount of CLA formed in vitro and in vivo (Kucuk *et al.*, 2001; Wang *et al.*, 2002). In contrast, other authors reported no changes in the proportion of CLA when concentrates were increased in diets (Loor *et al.*, 2004; Lee *et al.*, 2006). Recent studies have reported a relationship between the proportion of C_{18} fatty acids and their isomers and the forage-to-concentrate ratio (Laverroux *et al.*, 2011; Gudla *et al.*, 2012). Since the interactions of D × AO were significant in the content of palmitic, stearic, linoleic and α-linolenic acid, we can speculate that these differences may be caused by differences in the microbial populations developed during fermentation in vitro (Jalč *et al.*, 2002). As no interactions of D × AO occurred in the contents of the myristic and oleic acid, it seems that metabolic independence in the production of individual FA by microorganisms may have occurred. In the present experiment, the organic acids with oils affected the concentration of almost of all FA in batch cultures. In general, the diets supplemented with oils rich in linoleic acid increased the concentration of TVA and CLA in the rumen (Szöllöskei *et al.*, 2005; Szumacher-Strabel *et al.*, 2009). Of the three oil supplements used, SO contained the highest proportion of linoleic acid. The TVA concentration in batch cultures was highest with both the HF- and the HC-diets containing FUM+SO and MAL+SO, and it is evident that differences in TVA concentration were influenced by the level of linoleic acid in the oils added to the diets. Positive correlations between the amount of linoleic acid present in the diets of sheep and the TVA content in rumen fluid have been reported previously (Váradyová *et al.*, 2007). The combination of organic acid with LO in the present

experiment had no effect on the concentration of FA in batch cultures, in contrast to results reported an increase in duodenal flow of TVA and CLA after LO supplementation of high concentrate diets in cows (Loor *et al.*, 2004). Regarding the TVA and CLA concentration in batch cultures, the supplementation of the HF- and HC- diets with organic acids and plant oils was effective only for increasing TVA concentration, especially with RO and SO. These oils also contained higher proportions of oleic acid, which can form TVA for endogenous synthesis of CLA during BH to stearic acid. In addition, 95% of TVA is saturated to stearic acid after 5 h of incubation with the concentrate diet, compared with 78% with the forage diets (Laverroux *et al.*, 2011). It has been reported the increasing CLA production when incubated with fumarate (24 mmol/L), fish oil (24 mg) and safflower oil (120 mg) in vitro (Li *et al.*, 2011). This is in contrast to the results of the present work, in which organic acids with oils supplementation in comparison with the control did not increase CLA concentration. In the present experiment, the diets supplemented with organic acids and oils increased the production of TVA, resulting in the incomplete BH of oleic acid, which was decreased in both diets, and all supplements compared with the control. It is known that the C_{18} FA isomers, (including CLA and TVA) in ruminant products are mostly derived from incomplete BH of dietary UFA in the rumen (Fievez *et al.*, 2007). Recent study also showed that manipulating fermentation with linoleic acid (60 mg), fumaric acid (24 mmol/L) and malic acid (24 mmol/L) as propionate precursors affects BH by rumen microbes (Li *et al.*, 2010). These finding are consistent with the results of the present work, where the combined effect of organic acids with plant oils rich in linoleic acid influenced the BH of C_{18} FA. As the diets × organic acids with oils interaction occurred only in the BH of linoleic acid, the relationships between diets and organic acids can be presumed only in the BH of this fatty acid. It has been also suggested that fermentation of linoleic acid with organic acids acts as an alternative electron sink and may compete with methane generation and the BH of linoleic acid in the utilization of metabolic H_2 (Li *et al.*, 2010). On the other hand, no relationship between dietary concentration of α-linolenic acid and the level of BH was found (Doreau and Ferlay, 1994). In the present study, the average values of α-linolenic acid BH ranged from 78.6-90.9% for the HF-diet and from 77.5-98.4% for the HC-diet, respectively. These results are consistent with the results describing almost complete BH (85-100%) of α-linolenic acid in the rumen (Doreau and Ferlay, 1994).

In summary, we conclude from this study that the combination of organic acids and plant oils increased the concentration of TVA in the batch cultures with both HF-diet and the HC-diet, especially when SO was used; all three oils in combination with the organic acids were effective in decreasing the BH of oleic acid.

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