Influence of inoculated maize silage and sunflower oil on the *in vitro* fermentation, ciliate population and fatty acid outputs in the rumen fluid collected from sheep

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ABSTRACT: The effect of maize silage (MS) supplemented with sunflower oil (SO) on the rumen fermentation parameters, growth of ciliate population and fatty acid outputs was investigated for 72 hours using a batch culture fermentation technique. The rumen fluid from ruminally fistulated sheep was mixed with McDougall's buffer (1:1) and added (35 ml) to fermentation bottles containing 1.5 g (0.38 g of DM) of MS with or without SO (30 g/kg of DM). Four types of MS were used: uninoculated (MS) or inoculated with *Lactobacillus plantarum* CCM 4000 (MS+LP), *Lactobacillus fermentum* LF2 (MS+LF) or *Enterococcus faecium* CCM 4231 (MS+EF) in simultaneous incubations at 39 ± 0.5°C for 72 h *in vitro*. Total gas production was decreased by SO (by 16–17%) in MS and all inoculated MS. Methane production was not significantly influenced by SO. The concentration of total volatile fatty acids, molar proportions of acetate, propionate and *n*-butyrate were influenced by SO ($P < 0.05$; $P < 0.01$; $P < 0.001$). SO had no significant effect on the total ciliate number and growth of the examined ciliate species *Entodinium* spp., *Dasytricha ruminantium*, *Polyplastron multivesiculatum*, *Enoploplastron triloricatum* and *Diplodinium denticulatum*. The number of *Dasytricha ruminantium* with MS+LP was higher ($P < 0.01$) as compared to MS. Outputs of *trans* vaccenic acid (TVA), linoleic acid, conjugated linoleic acid (CLA) and α-linolenic acid were influenced by SO ($P < 0.05$; $P < 0.01$; $P < 0.001$). However, the output of CLA was increased only in MS+LF ($P < 0.001$). It can be concluded that the supplementation of sunflower oil into maize silage is not effective as dietary antiprotozoal agents in a short-time interval, but it might positively affect the rumen bacterial population and activities. Sunflower oil with inoculated and uninoculated maize silage could be effective for an increase of TVA in the rumen fluid of sheep.

Keywords: maize silage; inoculation; lipid metabolism; ciliate protozoa; *in vitro*

Ensiling is the process of feed conservation with a minimal loss of nutritive value by anaerobic fermentation of soluble carbohydrates to organic acids, preferably lactic acid, which reduces the pH (Saarisalo et al., 2007). Maize silage (MS) is a well digestible and palatable high-quality forage crop mainly used as a high energy feed silage for dairy cows. Starch in the kernels optimizes the growth of the rumen microbial population and influences the rate of microbial protein synthesis, nitrogen utiliza-

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tion and production of volatile fatty acids (Elizalde et al., 1999). Mechanical processing of whole maize plants after harvesting improves the digestion and utilization of MS and enhances ruminal fermentation because of more rapid attachment and more difficult colonization of rumen bacteria to the processed silage (Kozakai et al., 2007). Fresh whole maize dry matter containing 300–400 g/kg grains is rich in linoleic acid (LA; 550–620 g/kg of fatty acids; FA) and oleic acid (240–320 g/kg of FA) and poor in α-linolenic acid (< 20 g/kg of FA; Chilliard et al., 2007). Bacterial inoculants such as lactic acid producing bacteria are used as silage additives to potentiate the lactic acid production and to better preserve the ensiled material. Numerous studies dealt with the ensiling of maize without inoculants (Abdehadia et al., 2005; Kozakai et al., 2007) or with inoculants (e.g. *Lactobacillus plantarum*, *Lactobacillus buechneri, Propionibacterium acidipropionici*) (Filya, 2003; Filya and Sucu, 2007) or with commercial inoculants containing lactobacilli, enterococci, pediococci (Weinberg et al., 2004) or with mixtures of inoculants (Sucu and Filya, 2006; Koc et al., 2008). In addition, *in vitro* experiments have shown that microorganisms such as lactobacilli, lactococci, propionibacteriae, bifidobacteriae and enterococci are able to form conjugated linoleic acid (CLA) from linoleic acid in some growth media (Coakley et al., 2003; Sieber et al., 2004; Marciňáková, 2006). The survival and effect of three new probiotic inoculants (e.g. *L. plantarum* CCM 4000, *L. fermentum* LF2 and *E. faecium* CCM 4231) on the nutritive value and fermentation parameters of grass or maize silages was studied previously in our laboratory (Jalč et al., 2009a,b,c). A batch culture fermentation system showed the potential to appropriately simulate biohydrogenation of unsaturated fatty acids and production of C18:0 from unprotected FA sources (Fievez et al., 2007). Therefore, the possibilities to increase the concentration of polyunsaturated FA in MS using both microbial inoculants and plant oil were studied in the present work in batch cultures. Sunflower-seed oil was found to be an effective dietary supplement that produces a massive reduction in the rumen protozoa population (Ivan et al., 2001). A reduction in the rumen protozoa population should increase the rumen microbial synthesis of protein and, proportionally, reduce the requirement for dietary protein. Rumen ciliates can decrease the intestinal flow of amino acids, mainly those of bacterial origin, by 23–30% (Ivan et al., 2000). The aim of our study was to determine the influence of inoculated MS (i.e. *L. plantarum* CCM 4000, *L. fermentum* LF2, and *E. faecium* CCM 4231, those previously characterized in our laboratory) supplemented with sunflower oil on the fermentation parameters, ciliate population and FA outputs in batch culture fermentation.

MATERIAL AND METHODS

For the ensiling of whole maize plants (*Zea mays* L.) the following four treatments were used: untreated maize silage (MS) without inoculants; treated maize silage – inoculated by the *L. plantarum* CCM 4000 strain (MS+LP); treated maize silage – inoculated by the *L. fermentum* LF2 strain (MS+LF) and treated maize silage – inoculated by the *E. faecium* CCM 4231 strain (MS+EF). The inoculants were applied in a concentration of 10^9 CFU/ml in Ringer's solution (10 ml/kg of fresh whole plant maize). The counts of inoculants decreased during the ensiling of maize, and at the end of ensiling (105 days) the counts of inoculants were lower than 1.0 log10 CFU/g. The chemical composition of whole plant maize before ensiling was (g/kg DM): dry matter (DM): 288.3; crude protein (CP): 57.2; crude fibre (CF): 203.6; neutral detergent fibre (NDF): 515.5; acid detergent fibre (ADF): 216.4; lignin (sa) 30.1; fat: 22.8; ash: 53.6; organic matter (OM): 272.9. The nutrient composition and fermentation characteristics of uninoculated and three inoculated MS after 105 days of ensiling are shown in Table 1. The fatty acid composition of MS (i.e. uninoculated and inoculated after 105 days of ensiling) is documented in Table 2.

Three rumen-fistulated Merino sheep (4 years of age; 43 ± 3.0 kg) were used. Sheep were housed separately in pens and fed a diet consisting of 700 g/kg meadow hay and 300 g/kg barley grain with free access to water. Rumen fluid (RF) was collected about three hours after the morning feeding, transferred to the laboratory in a water bath preheated to 39 ± 0.5°C, squeezed through four layers of gauze, combined among sheep and purged with CO_2. The rumen ciliate population of sheep was A-type (*Polyplastron* and *Ophryoscolex* spp., Eadie, 1967).

The 120 ml serum bottles were used as fermentation vessels for the batch culture fermentation. RF was mixed with McDougall's buffer (McDougall, 1948) at a ratio of 1:1 and 35 ml of inocula was

Table 1. Nutrient composition and fermentation parameters in MS after 105 days of ensiling

	MS	MS+LP	MS+LF	MS+EF
DM (g/kg)	279.00	280.00	271.00	278.00
Ash (g/kg DM)	52.10	51.40	51.00	51.70
Crude protein (g/kg DM)	68.90	63.20	64.40	63.60
NDF (g/kg DM)	526.00	541.00	487.00	523.00
ADF (g/kg DM)	243.00	245.00	244.00	251.00
Fat (g/kg DM)	22.90	21.60	23.50	22.30
IVDMD (g/kg DM)	761.00	765.00	749.00	782.00
pH	3.44	3.48	3.50	3.54
Lactate (g/kg DM)	11.10	11.40	10.30	9.76
Acetate (g/kg DM)	1.68	1.85	4.60	1.82
Propionate (g/kg DM)	0.17	0.15	0.11	0.14
n-butyrate (g/kg DM)	ND	ND	ND	ND
Ammonia N (g/kg DM)	0.34	0.29	0.43	0.32
Inoculants (log10 CFU/g)	NI	< 1.00	< 1.00	< 1.00
ENT (log10 CFU/g)	< 1.00	< 1.00	< 1.00	< 1.00
LAB (log10 CFU/g)	1.10	< 1.00	< 1.00	< 1.00

MS = maize silage; DM = dry matter; NDF = neutral detergent fibre; ADF = acid detergent fibre; IVDMD = *in vitro* dry matter degradability; LP = *Lactobacillus plantarum* CCM 4000; LF = *Lactobacillus fermentum* LF2; EF = *Enterococcus faecium* CCM 4231; ENT = enterococci; LAB = lactic acid bacteria; NI = not isolated; ND = not determined
*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ express differences between control (MS) and inoculated MS

Table 2. Fatty acid composition of MS and sunflower oil (g/kg of FA) after 105 days of ensiling

	MS	MS+LP	MS+LF	MS+EF	SO
C12:0 lauric	3.40	3.91	5.62	5.23	0.43
C14:0 myristic	9.80	12.31	14.21	10.21	0.82
C16:0 palmitic	171.20	178.90	173.20	165.40	61.40
C16:1 palmitoleic	7.61	5.10	4.53	2.53	1.01
C18:0 stearic	74.20	81.90	78.60	84.40	31.20
C18:1*n*-9 oleic	232.40	176.70	172.80	149.60	326.10
C18:2*n*-6 linoleic	376.30	385.40	410.10	413.70	548.20
C18:3*n*-3 α-linolenic	63.10	88.30	77.10	88.70	11.00
C18:3*n*-6 γ-linolenic	2.20	1.22	2.80	3.42	0.01
C20:4 arachidonic	4.61	4.31	5.01	5.84	2.81

FA = fatty acids; MS = maize silage; SO = sunflower oil; LP = *Lactobacillus plantarum* CCM 4 000; LF = *Lactobacillus fermentum* LF 2; EF = *Enterococcus faecium* CCM 4231

pumped with an automatic pump into the preheated fermentation bottles containing 1.5 g (0.38 g of DM) of MS (uninoculated or inoculated). The fermentation bottles were filled up with CO_2, closed with a butyl rubber stopper and aluminum-sealed. Eight replicate fermentation bottles of MS were used for experimental groups (MS; MS+LP; MS+LF; MS+EF). Eight replicate fermentation bottles of MS were used for experimental groups with sunflower oil (SO; 30 g/kg of DM) additive (MS+SO; MS+LP+SO; MS+LF+SO; MS+EF+SO). 'Blank' fermentations (inoculum, no silage, no inoculants, and no additive) for the examination of fermentation activity of media were run simultaneously. The fatty acid composition of SO is shown in Table 2. The *in vitro* fermentation procedure and measurements were performed according to Váradyová et al. (2005). Methane concentration (percentage per 1 ml of gas volume) and volatile fatty acids (VFA) in the medium at the end of the fermentation period were determined by gas chromatography using a Perkin-Elmer Clarus 500 gas chromatograph (Perkin-Elmer, Inc., Shelton, CN, USA).

The samples for ciliate count were collected after 24 h. The strained rumen fluid from two replicate fermentation bottles of all treatment groups was fixed with an equal volume of 8% formaldehyde. The number of ciliate protozoa was counted microscopically according to the procedure described by Coleman (1978). Protozoa were identified according to Dogiel (1927) and Ogimoto and Imai (1981). The following rumen ciliate genera and species were established: *Entodinium* spp., *Dasytricha ruminantium*, *Isotricha* spp., *Ophryoscolex caudatus tricoronatus*, *Polyplastron multivesiculatum*, *Enoploplastron triloricatum*, and *Diplodinium denticulatum*.

Chemical analyses of MS after 105 days of ensiling were in triplicate (Table 1). Dry matter of MS was determined by oven drying at 103°C for 16 h. Dried (60°C, 48 h) samples were analysed for NDF and ADF according to Van Soest et al. (1991) using Fibertec 2010 (Tecator Comp., Höganäs, Sweden). NDF was assayed without heat stable amylase and expressed inclusive of residual ash. ADF is expressed inclusive of residual ash (Mertens, 2002). Standard methods were used for determining ash (AOAC, 1990 No. 942 05), N (AOAC, 1990, No. 968 06) and fat (AOAC, 1990, No. 983 23). A water extract of MS was prepared by adding deionized water to 20 g of MS to achieve a total of 300 g. The water extract was measured for pH, organic acids and ammonia N (AOAC, 1990 No. 920 03). Lactic acid and VFA (acetate, propionate, *n*-butyrate) were analysed by the Naumann and Bassler (1997) method.

Lipids from freeze-dried MS (after 105 days of ensiling) were extracted using the extraction-transesterification procedure of Sukhija and Palmquist (1988). The mixture of chloroform and methanol (2:1) was chosen as the extraction solvent. Extracted lipids were dissolved in 1 ml hexane with the internal standard tridecanoic acid (C13:0) and esterification of lipids was done with 2 ml 1N methanolic sodium methoxide (30 min, 50°C) and 3 ml 3N methanolic HCl (60 min, 50°C). After centrifugation at 200 × g for 5 min at laboratory temperature the samples as the upper hexane layers were used for the gas chromatographic analyses of methyl esters using a Hewlett-Packard 5890 gas chromatograph (Agilent Technologies, Inc., Santa Clara, CA, USA) equipped with a programmed 60 m HP-Innowa capillary column (180–240°C) and a flame ionization detector (FID).

FA outputs in uninoculated and inoculated MS with or without SO after 72 h of fermentation in RF were determined in lyophilized samples. The extraction and analysis of FA were performed as described by Váradyová et al. (2007). A 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 FID (constant flow, hydrogen 40 ml/min, air 400 ml, 260°C) was used for the determination of FA methyl esters. Analyses of FA (0.5 μl methyl esters in hexane injected at a 30:1 split ratio) were carried out under a temperature gradient (130°C for 1 min; 130–170°C at program rate 6.5°C/1 min; 170–206°C at program rate 1°C/min; 206–240°C at program rate 34°C/min) with H_2 as a carrier gas (flow 1.8 ml/min, velocity 44 cm/s, pressure 23.2 psi). The FA methyl ester peaks were identified with the commercial mixture (Supelco 37 component FAME MIX, Supelco, Bellefonte, PA, USA; *trans*11-vaccenic /TVA/ methyl ester, Supelco, Bellefonte, PA, USA; *cis*9, *trans*11 conjugated linoleic acid /CLA/, Matreya, PA, USA) and quantified by the internal standard of tridecanoic acid (C13:0, Supelco, Bellefonte, PA, USA).

The statistical analysis was based on analysis of variance (GraphPad Prism, GraphPad Software, Inc. San Diego, CA, USA) as a completely randomized design with 4 × 2 factorial arrangement of treatments that represent four MS groups (MS, MS+EF,

MS+LF, MS+LP) and two oil supplemental groups (without SO and with SO). Effects included silage (MS), oil (SO) and the interactions between MS and SO (MS × SO). Interactions between uninoculated and inoculated MS were analysed by two-way ANOVA with Bonferroni post-test. The differences between the treatment means were considered to be significant when $P < 0.05$.

RESULTS

After 72 hours of fermentation *in vitro* the SO supplementation decreased gas production in both uninoculated and inoculated MS (Table 3). The gas production of inoculated MS with SO was higher ($P < 0.01$) as compared to uninoculated MS. The concentration of total VFA ($P < 0.001$) and molar proportions of acetate ($P < 0.001$), propionate ($P < 0.05$) and *n*-butyrate ($P < 0.001$) were influenced by SO. The total VFA were lower ($P < 0.001$) with MS+EF *versus* MS. Compared to MS, higher molar proportions of acetate and *n*-butyrate were determined for MS+LF. The methane production was not significantly influenced by SO.

SO had no significant effect on the total ciliate number and individual ciliate species (Table 4). Interactions of MS and SO (MS × SO) in the number of *Isotricha* spp. and *Ophryoscolex caudatus tricoronatus* were detected ($P < 0.05$; $P < 0.01$). The number of *Dasytricha ruminantium* with MS+LP was higher ($P < 0.01$) as compared to MS.

Outputs of TVA ($P < 0.05$) and linoleic acid ($P < 0.001$) were influenced by SO (Table 5). The concentration of TVA of MS+EF was higher ($P < 0.01$) as compared to the MS. Outputs of CLA and α-linolenic acid were influenced by SO ($P < 0.01$; $P < 0.001$). However, interactions of MS and SO (MS × SO) in the outputs of CLA, α-linolenic and γ-linolenic were detected ($P < 0.001$; $P < 0.05$). Interactions of the MS and SO supplement (MS × SO) occurred also in the output of the majority of FA (i.e. myristic, palmitic, stearic, and oleic, respectively).

Table 3. Fermentation parameters of MS incubated in rumen fluid with either SO or without SO after 72 h of fermentation *in vitro*

Silage	Oil	Gas volume (ml/g DM)	Total VFA (mM)	Molar proportion of VFA			Methane (10^{-2} ml/ml)
				acetate	propionate	*n*-butyrate	
MS	none	240	38.5	562	256	103	7.76
	SO	200	35.2	551	242	92	7.12
MS+LP	none	250	39.3	574	240	114	7.36
	SO	210	36.4	543	233	100	7.26
MS+LF	none	245	40.1	588	237	119	7.86
	SO	210	36.5	579	228	106	6.10
MS+EF	none	245	35.6	576	238	109	7.29
	SO	210	32.3	565	222	101	7.02
SEM		2.1	0.93	5.9	7.2	4.4	0.532
Significance	MS	***	***	***	NS	*	NS
	SO	***	***	***	*	***	NS
	MS × SO	NS	NS	NS	NS	NS	NS
MS *vs.* MS+LP		**	NS	NS	NS	NS	NS
MS *vs.* MS+LF		**	NS	**	NS	NS	NS
MS *vs.* MS+EF		**	***	NS	NS	NS	NS

MS = maize silage; SO = sunflower oil; LP = *Lactobacillus plantarum* CCM 4000; LF = *Lactobacillus fermentum* LF 2; EC = *Enterococcus faecium* CCM 4231; NS = not significant; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$

Table 4. Ciliate number (*n*/ml) in rumen fluid incubated with maize silage (MS) with sunflower oil (SO) or without SO after 24 h incubation *in vitro*

Silage	Oil	Total count	*E.* spp	*Dr*	*I.* spp	*Oct*	*Pm*	*Et*	*Dd*
MS	none	180 850	143 400	10 180	1 700	1 230	850	310	80
	SO	167 200	154 700	8 840	1 350	710	780	240	120
MS+LP	none	181 000	158 450	11 900	1 230	1 050	980	290	120
	SO	153 850	134 400	10 650	1 380	930	610	170	110
MS+LF	none	184 200	168 300	10 250	1 340	650	890	170	70
	SO	189 300	164 250	12 250	1 700	930	890	220	150
MS+EF	none	178 950	166 300	9 540	1 280	540	790	210	150
	SO	199 050	184 850	8 800	730	640	530	180	130
SEM		10 391	12 530	776	154	113	99	39	21
Significance	MS	NS	NS	*	NS	**	NS	NS	NS
	SO	NS	NS	NS	NS	NS	NS	NS	NS
	MS × SO	NS	NS	NS	*	**	NS	NS	NS
	MS *vs.* MS+LP	NS	NS	NS	NS	NS	NS	NS	NS
	MS *vs.* MS+LF	NS	NS	**	NS	NS	NS	NS	NS
	MS *vs.* MS+EF	NS	NS	NS	NS	NS	NS	NS	NS

LP = *Lactobacillus plantarum* CCM 4000; LF = *Lactobacillus fermentum* LF 2; EC = *Enterococcus faecium* CCM 4231; *E.* spp = *Entodinium* spp.; *Dr* = *Dasytricha ruminantium*; *I.* spp = *Isotricha* spp.; *Oct* = *Ophryoscolex caudatus tricoronatus*; *Pm* = *Polyplastron multivesiculatum*; *Et* = *Enoploplastron triloricatum*; *Dd* =*Diplodinium denticulatum*; NS = not significant

*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$

Table 5. Output of FA (mg/g) in rumen fluid incubated with maize silage (MS) with sunflower oil (SO) or without SO after 72 h of fermentation *in vitro*

Silage	Oil	Fatty acids									
		myristic C14:0	palmitic C16:0	palmitoleic C16:1	stearic C18:0	oleic C18:1n-9	TVA	linoleic C18:2n-6	CLA	α-linolenic C18:3n-3	γ-linolenic C18:3n-6
MS	none	0.71	9.03	0.10	17.2	2.06	1.44	2.22	0.30	0.21	0.004
	SO	0.71	9.37	0.08	19.0	4.14	1.58	5.43	0.29	0.33	0.001
MS+LP	none	0.76	10.0	0.06	19.5	1.93	1.83	2.33	0.57	0.22	0.003
	SO	0.87	11.3	0.11	23.5	4.38	1.84	6.16	0.39	0.33	0.004
MS+LF	none	0.78	9.73	0.09	19.4	1.07	1.37	3.26	0.17	0.28	0.002
	SO	0.77	9.57	0.07	18.9	4.84	1.81	7.29	0.45	0.33	0.002
MS+EF	none	0.67	9.07	0.06	18.5	1.41	1.87	2.56	0.57	0.25	0.003
	SO	0.80	9.88	0.04	20.2	5.35	2.18	7.03	0.37	0.33	0.006
SEM		0.022	0.123	0.014	0.53	0.122	0.123	0.142	0.013	0.011	0.0012
Significance	MS	***	***	*	***	*	***	NS	***	*	NS
	SO	***	***	NS	***	***	*	***	**	***	NS
	MS × SO	**	***	NS	**	***	NS	NS	***	*	*
	MS *vs.* MS+LP	***	***	NS	***	NS	NS	NS	***	NS	NS
	MS *vs.* MS+LF	NS	NS	NS	NS	***	NS	NS	***	NS	NS
	MS *vs.* MS+EF	**	*	NS	NS	***	**	NS	***	NS	NS

LP = *Lactobacillus plantarum* CCM 4000; LF = *Lactobacillus fermentum* LF 2 = EC: *Enterococcus faecium* CCM 4231; TVA = *trans* vaccenic acid (*trans*11 C18:1); CLA = conjugated linoleic acid (*cis*9, *trans*11 C18:2)

NS = not significant

*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$

DISCUSSION

In our previous experiments, the effect of inoculated grass and maize silages on rumen fermentation and lipid metabolism in RUSITEC effluent was tested using the same inoculants (i.e. *L. plantarum* CCM 4000, *L. fermentum* LF2, and *E. faecium* CCM 4231). The inoculants used in grass silage were found to survive in higher counts contrary to low counts in maize silage (Jalč et al., 2009a,b). It is probably due to low pH in maize silage because the rumen pH has an important role in maintaining a viable environment suitable for microorganisms. It is known that the rumen pH near the neutral with a high amount of dietary linoleic acid modulates the reactions of biohydrogenation in a way that supports CLA and TVA contents in the rumen (Troegeler-Meynadier et al., 2003).

Starch in maize silage is a fermentable source of energy in contrast to grass silages where the main energy source is fermentable fibre. It is evident that the inoculants together with SO in the fermentation with MS could influence the fermentation of some products formed. In our experiment the *in vitro* gas production of MS (without SO) ranged from 240 to 250 ml/g DM. Chai et al. (2004) reported the gas production after 32 h of incubation with MS from 212 to 304 ml/g OM. SO decreased gas production (by 16–17%) in MS and all inoculated MS. Previous studies (Doreau et al., 1993; Jalč et al., 2006a) showed no effect or reduction of *in vitro* gas production in MS or forage diets with rapeseed, linseed, and fish oil. In our study, the molar proportion of VFA (i.e. acetate, propionate and *n*-butyrate) was influenced by SO. An increase of the molar proportion of propionate and a reduction of the molar proportion of acetate and *n*-butyrate with different oils, their blend and different diets were also reported previously (Doreau et al., 1993; Jalč et al., 2006b,c; Li and Meng, 2006). Recent studies showed that a blend of essential oils altered *in vitro* ruminal fermentation (Benchaar et al., 2007), however, it did not affect the fermentation or aerobic stability of maize silage (Kung et al., 2008). The methane production in the present study was not affected by SO treatment. The inhibitory effect of fat on rumen methanogenesis is determined by the source and quantity of fat used (Machmüller et al., 1998; Zhang et al., 2008).

In addition, 6% of SO in the diets of sheep substantially reduce protozoa in the rumen for over 84 days (Ivan et al., 2001). Unsaturated FA in plant oils are toxic to some rumen ciliates (Ivan et al., 2004) and reductions of protozoa as a predator of bacteria in the rumen would lead to an increase in bacterial biomass. The present results showed that the number of total ciliate population and *Entodinium* spp. was not affected by SO after 24 h incubation *in vitro*. Surprisingly the growth of *Dasytricha ruminantium* was increased by SO in the case of MS+LF. However, Kišidayová et al. (2006) suggested that the rumen ciliates had no uniform response to oil supplements in studies *in vitro*. In addition, recent studies showed that rumen ciliates play an important role in causing the high CLA and TVA concentration in RF (Devillard et al., 2006; Or-Rashid et al., 2007, 2008a).

Microorganisms used as inoculants for ensiling of MS in the present study are able to convert LA to CLA in some growth media (Marciňáková, 2006). LP added to grass silage diets was effective in increasing CLA concentrations and decreasing biohydrogenation of C18:2 and C18:3 while EF and LF had an opposite effect (Jalč et al., 2009a). The ensiling process does not reduce concentrations of PUFA and FA profiles in maize silage (Da Cruz et al., 2005; Ribeiro et al., 2005). Maize silage also has an impact on high TVA contents in RF of cattle (Or-Rashid et al., 2008b). Our results showed that SO after 72 h of incubation influenced the output of TVA (mainly MS+EF) and linoleic acid ($P < 0.001$), which could indicate a shift of the biohydrogenation of unsaturated FA to the accumulation of TVA. Li and Meng (2006) reported an influence on the biohydrogenation of unsaturated FA by addition of SO after 24 h incubation in RF *in vitro*. In addition, in our experiment MS × SO interaction was observed in the concentration of the majority of FA, relationships can be presumed in the outputs of these FA (i.e. myristic, palmitic, palmitoleic, stearic, oleic, CLA, α-linolenic and γ-linolenic; Table 5). It is known that in RF the accumulation of TVA is due to a surplus of free FA that inhibit the final hydrogenation of TVA to stearic acid (Gulati et al., 2000) or to the increased intake of substrates with a high concentration of linoleic acid in the diet (Beam et al., 2000) or to the interference of oleic acid with biohydrogenation of linoleic acid (Mosley et al., 2002). Therefore, we could suggest that dietary manipulation associated with addition of SO into the inoculated MS diets could reduce biohydrogenation in RF and might increase the postruminal flow of TVA. However, more studies are needed to determine these im-

pacts on the levels of CLA and TVA in meat and dairy products.

It can be concluded that the supplementation of sunflower oil into inoculated and uninoculated maize silage affected *in vitro* gas production and VFA concentration in 72 h batch cultures, however it did not affect the methane production and total ciliate number. The output of TVA was increased by SO supplementation especially in the case of MS+EF. The output of CLA was increased by SO only in MS+LF. Maize silage supplemented with sunflower oil seems not to be effective as dietary antiprotozoal agents in a short-time interval, but it might positively affect the rumen bacterial population and activities. Our results show the possibilities of increasing the concentration of polyunsaturated fatty acids in rumen fluid using plant oils as a supplement to silages inoculated with some microorganisms.

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