Acta Protozool. (2009) 48: 51–61

ACTA
PROTOZOOLOGICA

The Effects of Linoleic Acid on the Fermentation Parameters, Population Density, and Fatty-acid Profile of Two Rumen Ciliate Cultures, *Entodinium caudatum* and *Diploplastron affine*

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Summary. The effects of linoleic acid supplementation on protozoa counts, fermentation parameters and fatty-acid composition of *Entodinium caudatum* and *Diploplastron affine* were examined in *in vitro* cultures. *Entodinium caudatum* (EC) and *Diploplastron affine* (DA) were isolated from the rumen of sheep (Slovak Merino breed) and cultivated anaerobically in the presence of bacteria in caudatum-type medium. To test the effect of soluble linoleic acid (LA) on protozoan growth, both ciliate species were supplemented with LA (1.51 μg/L) on the day of dilution over a 30-day period. Twenty-four-hour fermentation parameters were examined on both ciliate cultures and their respective bacterial fractions. Ciliate counts of both EC and DA cultures were not significantly affected by supplemented LA. The major impact of the soluble form of LA supplement was found in the bacterial fractions of both ciliate cultures. LA supplementation had a greater effect on fermentation parameters and fatty-acid contents in the EC experimental groups than in the DA groups. Our results suggest that experimental rumen ciliates and their associated bacterial populations had different metabolic responses to the tested form and concentration of supplemented LA.

Key words: Protozoa, fatty acids, rumen fermentation parameters, *in vitro.*

Abbreviations: CLA – conjugated linoleic acids; DA – *Diploplastron affine*; EC – *Entodinium caudatum*; IVDMD – *in vitro* dry matter degradability; LA – linoleic acid; VFA – volatile fatty acids; SFA – saturated fatty acids; SCFA, short-chain fatty acids (C6:0–C10:0); MCFA – medium chain fatty acids (C12:0–C16:1); LCFA – long-chain fatty acids (>C17:0); MUFA – monounsaturated fatty acids; PUFA – polyunsaturated fatty acids.

INTRODUCTION

The rumen contains a complex community of microorganisms including bacteria, protozoa, fungi and bacteriophages, which create a specific ecosystem. This ecosystem is characterized by dynamic changes in microbial populations depending on diet composition. Up to 35% of the rumen microbial mass may be of protozoal origin (Williams and Coleman 1992), moreover, protozoa may also represent a significant source of unsaturated fatty acids, including conjugated linoleic acids (CLA) and vaccenic acid (VA), for the host animal

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(Devillard *et al*. 2006, Yáñez-Ruiz *et al*. 2006, Váradyová *et al*. 2008). CLA are naturally occurring fatty acids in foods obtained from ruminants. This group of compounds may be highly beneficial in improving human health. VA is a substrate for *de novo* synthesis of CLA in the mammary gland and animal tissues. On average, 7.5 to 15% of digested lipids in the rumen may be of protozoal origin (Keeney 1970). In a recently published paper, Yáñez-Ruiz *et al*. (2006) showed that from 30 to 50% of the unsaturated fatty acids, and 10 to 20% of the saturated fatty acids entering the duodenum come from protozoa. Other studies also suggested that protozoa might play a significant role as a source of unsaturated fatty acids absorbed in the ruminant gut. Protozoa cells may protect unsaturated fatty acids from biohydrogenation by incorporating them into structural phospholipids (Yáñez-Ruiz *et al*. 2007). Rumen protozoa are richer in some unsaturated fatty acids than bacteria (Viviani 1970, Devillard *et al*. 2006, Váradyová *et al*. 2008). Or-Rashid *et al*. (2007) showed that the protozoal fraction had 3.7, 4.8, and 4.3 times higher contents of oleic, linoleic and linolenic acids, respectively, than the bacterial fraction. Some authors claimed that linoleic acid may originate from feed particles collected by protozoa (Viviani *et al*. 1968) or may be synthesized by protozoa from ^{14}C-acetate (Or-Rashid *et al*. 2007). In our previous study (unpublished), we observed that the fatty-acid profile may depend on the ciliate species. In that study, three species of ciliates (*Diploplastron affine*, *Eudiplodinium maggii*, *Entodinium caudatum*) were examined for the content of long-chain fatty acids. The highest levels of total C18:1 (24.86% FAME) and C18:2 (10.92% FAME) were observed in *Diploplastron affine,* whereas C18:3 (5.19% FAME) in *Eudiplodinium maggii*. Fatty acids of feed or diet supplements may influence the rumen fermentation pattern. It is well known that coconut oil, which is rich in C12:0 and C14:0, negatively affects microbial populations, especially of protozoa (Machmüller and Kreuzer 1999, Soliva *et al*. 2004, Machmüller 2006, Cieślak *et al*. 2006a). Doreau and Ferlay (1995) showed that adding lipids had very variable effects on protozoa counts. Unsaturated fatty acids usually influence protozoa counts by exerting a toxic effect (Newbold and Chamberlain 1988, Ivan *et al*. 2003, Hristov *et al*. 2004, Baah *et al*. 2007). On the other hand, little information is available on the stimulatory effect of particular long-chain fatty acids on ruminal protozoa (Kišidayová *et al*. 2005, Cieślak *et al*. 2006b, Kišidayová *et al*. 2006). *Entodinium caudatum* and *Dipoplastron affine* are common rumen ciliates in domesticated ruminants. They are representatives of two major metabolic rumen ciliate populations, amylolytic (*Entodinium caudatum*) and cellulolytic (*Diploplastron affine*) ones and therefore they were selected to examine the influence of linoleic acid supplementation on protozoa counts, fermentation parameters and fatty acid composition of *in vitro* cultures.

MATERIAL AND METHODS

Ciliates and culture conditions

Entodinium caudatum (EC) and *Diploplastron affine* (DA) were isolated from the rumen of sheep (Slovak Merino breed). Ciliates were cultivated anaerobically (in 20 ml of medium in glass tubes with screw caps, Schott, Germany) in the presence of bacteria according to Williams and Coleman (1992) and Coleman (1978) in caudatum-type medium supplemented with 10% (v/v) autoclaved rumen fluid (autoclaving following removal of ciliates by centrifugation), microelement solution – 1ml/L, and D-glucose – 0.4 g/L, proline – 0.115 g/L (Sigma), betaine – 0.153 g/L (Sigma), insulin – 17.4 nmol/L (Sigma, Kišidayová *et al*. 2000). *Entodinium caudatum* was fed daily with a mixture of ground wheat gluten (0.025g/L, Sigma), β-sitosterol (Sigma)-covered rice starch (0.025 g/L, BDH Chemicals Ltd. Poole, England) and finely ground leaves of *Dactylis glomerata* (0.05 g/L). *Diploplastron affine* was fed daily with a mixture of finely ground wheat gluten (0.006g/L), maize starch (0.006g/L, Maizena, Dr. Oetker s.r.o, Bratislava), leaves of *Dactylis glomerata* (0.013 g/L), and crystalline cellulose (0.015 g/L, Sigmacell 20).

The cultures were diluted with an equal volume of fresh medium every 4th–5th day of cultivation. The culture medium consisted of, (g L^{-1}): K_2HPO_4 – 6.35, KH_2PO_4 – 5.0, NaCl – 0.65, $MgSO_4 \times 7\ H_2O$ – 0.09, $CaCl_2$ (dried) – 0.045, CH_3COONa – 0.75, and neutralized L-cysteine hydrochloride 0.2, and bubbled with 5% CO_2 in nitrogen (Kišidayová *et al*. 2000).

Long-term linoleic acid treatment

As our preliminary cultivation studies showed, the small increase of water-soluble linoleic acid (3 μg/L) can decrease the growth of ciliates *in vitro* (Kišidayová *et al*. 2005), hence in presented research we supplemented each of the experimental ciliate species with dose of 1.51 μg/L of LA (C18:2; linoleic acid-water soluble, Sigma) on the day of dilution over a 30-day period. Parallel control cultures of each of the ciliate species were kept in the absence of LA. Cultivation conditions were as described above. Long-term LA-supplemented cultures were used in fermentation experiments in groups supplemented with LA.

Fermentation experiments

Fermentation incubations (24 h) were carried out in 50 ml glass syringes (Sigma, St. Louis, MO, USA) under anaerobic conditions. The cultures were allowed to grow for 5 days after dilution before starting experimental fermentation incubations. The experiment was

carried out in 5 parallel glass syringes per group and repeated once (n = 10) according to Kišidayová *et al*. (2000). Axenic cultivation of rumen ciliates has not been successful or developed yet. To distinguish the effect of LA on protozoan and the effect on bacterial cells, each experimental group was divided into two subgroups: whole protozoan culture (protozoa plus bacteria) and bacterial fraction (bacteria without protozoa). The bacteria were separated from protozoa by centrifugation at 400 *g* for 2 min. and the supernatant was used as the bacterial fraction. The experimental groups consisted of control (no LA) and long-term LA treated groups. The inocula (35 ml) were added to the syringes together with feed substrates. The amounts and composition of feed substrates for fermentation experiments were identical with basic cultures. The fatty acid composition (% of total fatty acids) of *Dactylis glomerata* used in both cultures was as follows: C12:0 lauric – 1·11, C14:0 myristic – 1.58, C16:0 palmitic – 44.32, C18:0 stearic – 4.48, C18:1 *c*9 oleic – 5.78, C18:2 *c*9*c*12 linoleic – 11.97, C18:3 *c*9*c*12*c*15 linolenic – 18.04 and other fatty acids – 12.72. LA (1.51 µg/L) was added according to the treatment groups. Throughout the incubation period (24 h), the temperature in the incubator was maintained at 39°C. The experimental design for both ciliate species is illustrated below:

1. Ciliates + bacteria – control group (DA, EC, respectively).
2. Ciliates + bacteria with LA (DA + LA, EC + LA, respectively).
3. Bacterial fraction of the ciliates – control group.
4. Bacterial fraction of the ciliates with LA.

Laboratory analysis and statistical evaluation

Gas production was measured by a syringe method (Váradyová *et al*. 2005). At the end of the incubation the gas was removed from the syringes by means of gas-tight syringes (2 ml) and analyzed by gas chromatography (Perkin-Elmer Clarus 500, Perkin-Elmer, Inc., Shelton, CN, USA). The methane content was expressed per ml of gas volume produced. After cooling, the contents of syringes were transferred into tubes and centrifuged at 3500 *g* for 10 min. Recovery of metabolic hydrogen (2Hrec, %) was calculated using the following equations:

$$2Hrec, \% = (4M + 2P + 2B + 4V)/(2A + P + 4B + 3V) \times 100,$$

where M, P, B, V, A are the molar amounts of methane, propionate, butyrate, valerate, and acetate, respectively (Demeyer 1991, Váradyová *et al*. 2006).

The residues were washed twice with distilled water, centrifuged and dried to a constant weight at 105°C (Mellenberger *et al*. 1970) to assess *in vitro* dry matter degradability (IVDMD, Kišidayová *et al*. 2000). The concentration of VFA after 24 h fermentation experiments was quantified by gas chromatography (VARIAN CHROMAPACK, CP-3380) according to Tangerman and Nagengast (1996). Analysis of fatty acid methyl esters (FAME) in experimental groups after 24 h fermentation experiments was performed using a gas chromatograph (VARIAN CHROMAPACK, CP-3380) equipped with a flame ionisation detector (FID) and Chrompac CP-Sil 88 column (100 m, 0.25 mm, 0.2 µm film thickness, Varian). Ultra-high-purity helium was used as the carrier gas at the constant flow of 30.0 mL/min. 2 µl of each sample was injected in splitless mode. The splitting ratio to the flame ionization detector was 1:90. The reaction temperatures were programmed as follows: initial 145°C for 9 min., then increasing at 4°C/min. to 240°C (Cieślak *et al*. 2009). Fatty acid peaks were quantified by reference to the internal standard and identified by comparison with the retention times of known standards (37 FAME Mix, Supelco, Poole, England and C18:2 *c*9*t*11, Matreya, Pleasant Gap, PA, USA). The fatty acid profile was expressed as a percentage of total fatty acids. Ammonia was quantified spectrophotometrically using the Nessler reagent as described by Szumacher-Strabel *et al*. (2002). Samples for counts were collected after 24 h fermentation and fixed with equal volumes of 8% formaldehyde. Fixed ciliates were counted under a light microscope according to Coleman (1978).

Statistical analysis was performed by analysis of variance (GraphPad Prism, GraphPad Software, Inc. San Diego, CA, USA) using a 2 × 2 factorial design that represents two experimental groups (*Entodinium caudatum*, *Diploplastron affine* and respective bacterial fractions of both protozoans) and LA treatment (without and with LA). Interactions between control and experimental groups were analysed by two-way ANOVA with the Bonferroni post-test. Ciliate numbers were analyzed by the t-test. The differences between treatment means were considered to be significant when $P < 0.05$.

RESULTS

Effects of linoleic acid on fermentation parameters and ciliate growth

LA supplementation was found to affect the ammonia concentration of both EC experimental groups ($P < 0.05$, Table 1). A relationship was found between the EC experimental groups and LA treatment (S × EG) in respect to total gas ($P < 0.001$, Table 1). The propionate concentration in the EC bacterial group decreased to the respective control value ($P < 0.05$). Interaction of DA experimental groups and LA treatment (S × EG) occurred in IVDMD ($P < 0.001$, Table 2), and the P values are hard to interpret. Methane production, total VFA, concentrations of acetate and butyrate in the DA groups were not affected by LA supplementation. Iso-butyrate and iso-valerate concentrations of the EC experimental groups were not influenced by LA supplementation (Table 3). Interaction of the EC experimental groups and LA supplementation (S × EG) occurred in respect to valerate concentration ($P < 0.01$) and hydrogen recovery ($P < 0.05$, Table 3). The acetate to propionate ratio (A:P) was increased in the EC bacterial group ($P < 0.001$, Table 3). Iso-butyrate, iso-valerate, valerate, hydrogen recovery, A:P ratio of the DA experimental groups were not influenced by LA supplementation (Table 4). Ciliate numbers of both EC and DA cultures were not significantly affected by LA supplementation.

Table 1. Fermentation parameters of *Entodinium caudatum* culture treated with linoleic acid.

Experimental group (EG)	Supplement (S)	Ammonia (mmol/l)	IVDMD (%)	Total gas (ml)	Methane (10^{-2} ml/ml)	Total VFA (mmol/l)	Acetate (mol/mol)	Propionate (mol/mol)	Butyrate (mol/mol)
EC+ bacteria	Control	10.43	97.16	2.95	3.84	30.67	62.06	25.95	9.99
	LA	8.54	98.28	2.25	4.25	30.01	62.48	24.04	10.39
Bacterial fraction of EC	Control	10.95	97.04	1.75	1.61	37.50	67.88	24.04	6.63
	LA	7.11	97.77	1.80	1.32	40.80	71.84	20.86	5.87
S.E.M.		1.17	1.55	0.16	0.64	1.33	1.35	0.82	0.50
Significance	S	*	ns	***	ns	ns	ns	**	ns
	S × EG	ns	ns	***	ns	ns	ns	ns	ns
EC: Control *vs.* LA		ns	ns	–	ns	ns	ns	ns	ns
EC bacterial fraction: Control *vs.* LA		ns	ns	–	ns	ns	ns	*	ns

EC – *Entodinium caudatum*; LA – linoleic acid; ns – not significant; IVDMD – *in vitro* dry matter degradability; VFA – volatile fatty acids; *P < 0.05; **P < 0.01; ***P < 0.001.

Table 2. Fermentation parameters of *Diploplastron affine* culture treated with linoleic acid.

Experimental group (EG)	Supplement (S)	Ammonia (mmol/l)	IVDMD (%)	Total gas (ml)	Methane (10^{-2} ml/ml)	Total VFA (mmol/l)	Acetate (mol/mol)	Propionate (mol/mol)	Butyrate (mol/mol)
DA + bacteria	Control	7.84	66.02	2.17	3.29	20.39	53.44	29.57	14.08
	LA	6.97	89.05	2.45	3.95	20.63	54.73	29.13	13.60
Bacterial fraction of DA	Control	8.66	46.87	2.20	0.82	21.43	55.81	28.13	13.52
	LA	7.68	45.38	2.15	0.68	18.54	52.91	30.17	15.01
S.E.M.		1.17	1.55	0.16	0.64	1.33	1.35	0.82	0.50
Significance	S	ns	***	ns	ns	ns	ns	ns	ns
	S × EG	ns	***	ns	ns	ns	ns	ns	ns
DA: Control *vs.* LA		ns	–	ns	ns	ns	ns	ns	ns
DA bacterial fraction: Control *vs.* LA		ns	–	ns	ns	ns	ns	ns	ns

DA – *Diploplastron affine*; LA – linoleic acid; ns – not significant; IVDMD – *in vitro* dry matter degradability; VFA – volatile fatty acids; *P < 0.05; ** P < 0.01; *** P < 0.001.

Table 3. Proportion of some individual fatty acids, acetate: propionate ratio, hydrogen recovery and protozoan number of *Entodinium caudatum* culture treated with linoleic acid.

Experimental group (EG)	Supplement (S)	Iso-butyrate (mol/mol)	Iso-valerate (mol/mol)	Valerate (mol/mol)	Acetate: propionate	Hydrogen recovery (%)	Protozoan number (1 ml^{-1})
EC+ bacteria	Control	0.66	0.72	0.62	2.39	56.74	16260
	LA	1.37	0.87	0.08	2.61	68.27	15640
Bacterial fraction of EC	Control	1.25	0.20	0.0	2.83	39.40	–
	LA	1.15	0.28	0.0	3.44	37.66	–
S.E.M.		0.26	0.08	0.11	0.10	2.76	770
Significance	S	ns	ns	**	***	ns	ns
	S × EG	ns	ns	**	ns	*	
EC: Control *vs.* LA		ns	ns	–	ns	–	
EC bacterial fraction: Control *vs.* LA		ns	ns	–	***	–	

EC – *Entodinium caudatum*; LA – linoleic acid; ns – not significant; * P < 0.05; ** P < 0.01; *** P < 0.001.

Table 4. Proportion of some individual fatty acids, acetate: propionate ratio, hydrogen recovery and protozoan number of *Diploplastron affine* culture treated with linoleic acid

Experimental group (EG)	Supplement (S)	Iso-butyrate (mol/mol)	Iso-valerate (mol/mol)	Valerate (mol/mol)	Acetate: propionate	Hydrogen recovery (%)	Protozoan number (1 ml^{-1})
DA+ bacteria	Control	1.40	0.41	1.11	1.84	46.81	70.60
	LA	1.01	0.41	1.11	1.88	39.92	97.68
Bacterial fraction of DA	Control	1.03	0.36	1.15	2.00	27.52	–
	LA	0.51	0.32	1.08	1.78	25.81	–
S.E.M.		0.26	0.08	0.11	0.10	2.76	10
Significance	S	ns	ns	ns	ns	ns	ns
	S × EG	ns	ns	ns	ns	ns	
DA: Control *vs.* LA		ns	ns	ns	ns	ns	
DA bacterial fraction: Control *vs.* LA		ns	ns	ns	ns	ns	

DA – *Diploplastron affine*; LA – linoleic acid; ns – not significant.

Effects of linoleic acid on fatty acid proportions of ciliate cultures

In our study, it was difficult to distinguish protozoan fatty acids because of the presence of bacterial populations. No separation of protozoa from bacteria was carried out due to the low concentration of ciliate cells for FA analysis in the fermentation experiments. The fatty-acid composition in both cultures is summarized in Tables 5 to 8. In both of the analyzed cultures, the predominant fatty acids were palmitic (C16:0) and stearic (C18:0) acids. Interactions of the EC experimental groups and LA supplementation (S × EG) in respect to the content of SFA ($P < 0.05$) and SCFA ($P < 0.01$) were detected, but the P values are hard to interpret (Table 5). The contents of MCFA, LCFA, and C18:1 oleic acid were not influenced by LA supplementation in the EC experimental groups. The content of *cis* C18:1 oleic acid in the EC experimental groups was affected by LA treatment ($P < 0.05$). Compared with the control, lower concentrations of palmitic acid ($P < 0.05$) were found in the EC bacterial group. The concentration of stearic acid in both EC experimental groups was lower ($P < 0.001$) as compared with the control. An interaction of the DA experimental groups and LA supplement (S × EG) in terms of palmitic content acid was detected, but the P value is hard to interpret ($P < 0.05$, Table 6). The stearic acid content was decreased in the DA bacterial group ($P < 0.01$). Interactions of EC experimental groups and LA supplementation (S × EG) occurred in terms of the contents of linolenic acid ($P < 0.01$) and omega-3 fatty acids ($P < 0.05$, Table 7). LA supplementation increased the contents of linoleic acid, PUFA, and omega-6 acids ($P < 0.01$) in the EC bacterial fraction (Table 7). No effects of LA supplementation on C18:1 *trans* oleic, C18:2 linoleic, C18:3 linolenic, MUFA, PUFA, CLA *c*9*t*11, omega-6, and omega-3 fatty acids were detected in the DA experimental group (Table 8).

DISCUSSION

Effects of linoleic acid on ciliate number

Oils rich in saturated fatty acids or unsaturated fatty acids used as a dietary fat may limit the number of ruminal protozoa both *in vivo* and *in vitro*. It has been shown that among C18 fatty acids, linoleic acid exerts the most pronounced toxic effect on protozoan populations (Girard and Hawke 1978; Newbold and Chamberlain 1988; Ivan *et al*. 2001; Hristov *et al*. 2003, 2004; Baah *et al*. 2007; Dayani *et al*. 2007). Several oils (sunflower, safflower and soybean) were studied as sources of LA to increase CLA concentrations in ruminant products. Their effects depend, among others, on the type of diet. For example, experiments with a barley silage-based diet and sunflower seed oil (6% of dietary dry matter) showed that *Isotrichids* (*Isotricha* and *Dasytricha* spp.) and cellulolytic ciliates (*Polyplastron, Diplodinium* and *Enoploplastron*) are more sensitive to LA than *Entodin-*

Table 5. Fatty acid composition (% of total fatty acids) of *Entodinium caudatum* culture treated with linoleic acid.

Experimental group (EG)	Supplement (S)	SFA	SCFA	MCFA	C16:0 palmitic	LCFA	C18:0 stearic	C18:1 oleic	C18:1 *cis* oleic
EC+ bacteria	Control	72.68	5.60	37.02	22.26	57.38	29.17	8.19	6.91
	LA	68.97	11.20	37.15	19.56	51.66	17.85	10.18	9.12
Bacterial fraction of EC	Control	72.24	5.56	33.49	22.44	60.95	32.15	9.61	8.69
	LA	60.55	6.02	33.66	18.92	60.32	18.45	15.82	14.60
S.E.M.		1.83	0.77	1.67	1.02	2.05	1.17	2.32	1.89
Significance	S	***	***	ns	**	ns	***	ns	*
	S × EG	*	**	ns	ns	ns	ns	ns	ns
EC: Control *vs.* LA		–	–	ns	ns	ns	***	ns	ns
EC bacterial fraction: Control *vs.* LA		–	–	ns	*	ns	***	ns	ns

EC – *Entodinium caudatum*; LA – linoleic acid; ns – not significant; SFA – saturated fatty acids; SCFA – short-chain fatty acids (C6:0–C10:0); MCFA – medium-chain fatty acids (C12:0–C16:1); LCFA – long-chain fatty acids; * P < 0.05; ** P < 0.01; *** P < 0.001.

Table 6. Fatty acid composition (% of total fatty acids) of *Diploplastron affine* culture treated with linoleic acid.

Experimental group (EG)	Supplement (S)	SFA	SCFA	MCFA	C16:0 palmitic	LCFA	C18:0 stearic	C18:1 oleic	C18:1 *cis* oleic
DA+ bacteria	Control	68.69	3.29	35.65	23.55	61.06	22.81	13.55	11.23
	LA	67.38	3.72	35.51	22.31	60.77	20.18	13.54	11.20
Bacterial fraction of DA	Control	68.84	3.58	38.29	25.20	58.13	23.98	13.66	11.81
	LA	68.08	4.58	33.42	19.49	62.0	18.18	11.37	10.39
S.E.M.		1.83	0.77	1.67	1.02	2.05	1.17	2.32	1.89
Significance	S	ns	ns	ns	**	ns	***	ns	ns
	S × EG	ns	ns	ns	*	ns	ns	ns	ns
DA: Control *vs.* LA		ns	ns	ns	–	ns	ns	ns	ns
DA bacterial fraction: Control *vs.* LA		ns	ns	ns	–	ns	**	ns	ns

DA – *Diploplastron affine*; LA – linoleic acid; ns – not significant; SFA – saturated fatty acids; SCFA – short-chain fatty acids (C6:0–C10:0); MCFA – medium-chain fatty acids (C12:0–C16:1); LCFA – long-chain fatty acids (> C17:0); * P < 0.05; ** P < 0.01; *** P < 0.001.

Table 7. Composition of fatty acids and their isomers (% of total fatty acids) of *Entodinium caudatum* culture treated with linoleic acid.

Experimental group (EG)	Supplement (S)	C18:1 *trans* oleic	C18:2 linoleic	C18:3 linolenic	MUFA	PUFA	*c*9*t*11-CLA	omega-6	omega-3
EC+ bacteria	Control	1.28	6.87	4.70	15.52	11.81	0.15	6.14	4.97
	LA	1.06	8.02	6.70	16.31	14.73	0.19	6.86	6.70
Bacterial fraction of EC	Control	0.92	6.34	4.37	16.60	11.16	0.24	5.59	4.76
	LA	1.22	11.03	4.45	22.97	16.48	0.36	10.37	4.74
S.E.M.		0.70	1.04	0.43	1.97	1.23	0.07	1.01	0.43
Significance	S	ns	**	*	ns	**	ns	**	ns
	S × EG	ns	ns	*	ns	ns	ns	ns	*
EC: Control *vs.* LA		ns	ns	–	ns	ns	ns	ns	–
EC bacterial fraction: Control *vs.* LA		ns	**	–	ns	**	ns	**	–

EC – *Entodinium caudatum*; LA – linoleic acid; ns – not significant; MUFA – monounsaturated fatty acids; PUFA – polyunsaturated fatty acids; * P < 0.05; ** P < 0.01.

Table 8. Composition of fatty acids and their isomers (% of total fatty acids) of *Diploplastron affine* culture treated with linoleic acid.

Experimental group (EG)	Supplement (S)	C18:1 *trans* oleic	C18:2 linoleic	C18:3 linolenic	MUFA	PUFA	*c*9*t*11-CLA	omega-6	omega-3
DA+ bacteria	Control	2.32	6.86	2.45	21.85	9.47	0.22	5.81	2.59
	LA	2.34	7.76	2.92	21.71	10.91	0.18	6.53	3.14
Bacterial fraction of DA	Control	1.86	6.33	2.79	21.83	9.33	0.15	5.72	2.92
	LA	0.99	9.45	3.23	19.10	12.82	0.38	8.14	3.35
S.E.M.		0.70	1.04	0.43	1.97	1.23	0.07	1.01	0.43
Significance	S	ns	ns	ns	ns	ns	ns	ns	ns
	S × EG	ns	ns	ns	ns	ns	ns	ns	ns
DA: Control *vs.* LA		ns	ns	ns	ns	ns	ns	ns	ns
DA bacterial fraction: Control *vs.* LA		ns	ns	ns	ns	ns	ns	ns	ns

DA – *Diploplastron affine*; LA – linoleic acid; ns – not significant; MUFA – monounsaturated fatty acids; PUFA – polyunsaturated fatty acids.

ium species (Ivan *et al*. 2001). In contrast, Váradyová *et al*. (2007) showed that 5% of the dietary dry matter of sunflower seed oil supplements to a hay and barley diet (80:20) decreased only *Isotricha* spp. and *Eremoplastron dilobum* populations in sheep rumen. On the other hand, a positive effect of 5% evening primrose oil (containing 81% of LA) on *Entodinium* spp. and *Diploplastron affine* populations in an artificial rumen with a diet consisting of meadow hay and ground barley (60:40) was observed (Kišidayová *et al*. 2006). Evening primrose oil increased the population of *Entodinium* spp. and *Diploplastron affine* by 32 and 21%, respectively, in contrast to reducing populations of *Dasytricha ruminantium*, *Eremoplastron dilobum*, *Polyplastron multivecsiculatum*, and *Isotricha* spp. However, it is difficult to compare the results of studies dealing with different sources of LA and different diets (Cieślak *et al*. 2006a).

Our results suggest that experimental rumen ciliates and their associated bacterial populations had different metabolic responses to the tested form and concentration of supplemented LA. In respect to the majority of the studied parameters, the soluble form of LA affected only the bacterial fractions of protozoa. We suggested that tested soluble form of LA was available only for bacteria. It is well known that rumen ciliates preferred particular forms of substrates and supplements (Hino *et al*. 1973, Williams and Coleman 1992). The presented data may also be explained by the low level of linoleic acid supplementation (1.5 µg/L). However, linoleic acid at a low concentration can be a stimulatory factor in cell physiology (Czerkawski *et al*. 1975, Mackie and White 1990, Murga *et al*. 2000, Kišidayová *et al*. 2005). Cellular lipids of rumen microorganisms may be generated either by *de novo* synthesis, or by the direct incorporation of performed precursor molecules, which may come from diet (Harfoot and Hazlewood 1997). Nonetheless, Kišidayová *et al*. (2005) reported that at a concentration of 3 µg/L soluble linoleic acid already inhibited *Diplodinium* and *Entodinium* growth in *in vitro* cultures.

Effects of linoleic acid on culture fermentation parameters

Our results suggest that experimental rumen ciliates and their associated bacterial population had different metabolic responses to the tested form and concentration of the LA supplement. The major effects of supplementing soluble LA were observed in the EC bacterial fraction in contrast to its weak effect on DA experimental groups. However, the detected interactions in experimental groups, especially of EC cultures, point to the close metabolic relationship between bacteria and protozoa. The bacteria associated with EC were fully capable of degrading the experimental substrates in contrast to the strong interaction in IVDMD (S × EG) in DA culture. The lower IVDMD in the DA bacterial fraction was probably caused by the presence of undigested crystalline cellulose. These results indirectly point to the ability of DA to degrade crystalline cellulose. The higher

IVDMD in the DA culture, after LA supplementation, can probably be explained by the direct impact of LA on metabolic activities. These findings are consistent with our previous study on the effect of LA on cryopreservation of several rumen ciliate species (Kišidayová *et al*. 2005). Similar effects of LA were observed in *in vitro* studies of other authors (Dohme *et al*. 2001, Hristov *et al*. 2004). A strong interaction between EC groups and LA supplementation was detected total gas production in contrast to no effect of the LA supplement on DA groups. Although rumen ciliates have an important role in rumen methane production (Newbold *et al*. 1995), methanogenesis in experimental ciliate cultures was not affected by LA supplementation, though a higher LA dose may decrease methane production (Dohme *et al*. 2001; McGinn *et al*. 2004; Cieślak *et al*. 2006a; Jalč *et al*. 2006, 2007). Likewise, in a study by Cieślak *et al*. (2006b), 7% of rapeseed, linseed or sunflower oils supplemented to diets tested in a Rusitec system did not reduce methane release (mmol/d) or protozoa count. Rumen protozoa may contribute from 30 to 46% of VFA production (Michałowski 1987). Effect of supplemented fatty acid on volatile fatty acids concentration in mixed rumen protozoa culture depends on type of fatty acid. Ajisaka *et al*. (2002) reported no effect on total VFA and increased molar proportion of propionate with addition of C8:0 and C10:0 FA. In the present study, total volatile fatty acids were not affected by linoleic acid in either *Entodinium caudatum* or *Diploplastron affine* groups. This is consistent with other studies (Dohme *et al*. 2001, Hristov *et al*. 2004, Devillard *et al*. 2006). Ivan *et al*. (2000) observed that the type of diet or fauna did not affect the total volatile fatty acid concentration in rumen fluid. As we cannot distinguish the bacterial and protozoal origin of VFA, it is very difficult to discuss the changes in the proportions of particular VFA between the experimental groups. Addition of linoleic acid to the EC bacterial fraction reduced the propionate concentration and elevated the acetate-to-propionate ratio. These results are in contrast to those of Ivan *et al*. (2001), who reported a significant increase in the propionate concentration. The DA culture response was similar to that of *Eremoplastron dilobum* cultures to unsaturated fatty acids from plant oils in total and particular VFA (Cieślak *et al*. 2006b). EC contains higher than DA concentration (not presented data) of odd- and branch-chained fatty acids that play role in H transfer, thus conserving potential energy (Or-Rashid *et al*. 2007). The interaction of EC experimental groups and LA supplementation in hydrogen recovery was detected, but different responses of DA groups were observed, because LA supplementation did not affect hydrogen recovery. However, the obtained results are still hard to interpret.

Effects of linoleic acid on the fatty-acid composition of the cultures

Differences in the effects of LA on fatty-acid content between *Diploplastron affine* and *Entodinium caudatum* are probably indicative of the varied roles of individual rumen ciliate species in rumen fatty-acid metabolism. Our results on the predominant fatty acids in whole cultures (C16:0 and C18:0) are consistent with other studies (Viviani 1970, Czerkawski 1976, Or-Rashid *et al*. 2007, Váradyová *et al*. 2008). These findings are also in agreement with work of Or-Rashid *et al*. (2007) who stated the predominant role of palmitic and stearic acids in both the bacterial and protozoal fractions. In our research we found the effect of linoleic acid on concentration either of C16:0 or C18:0 but the LA supplement decreased palmitic and stearic acid contents only of DA experimental groups. As an interaction occurred between the DA experimental groups and LA supplementation in terms of the palmitic acid content, a relationship in the production of palmitic acid between both DA experimental groups can be presumed. On the other hand, the content of stearic acid in DA cultures was influenced only by the DA bacterial fraction after LA supplementation. This is in contrast to effect of LA on the content of the palmitic and stearic acids in EC experimental groups. Although both fatty acids decreased, no interactions were detected in their production. LA supplementation lowered the concentration of both fatty acids, especially in the EC bacterial fraction. We can speculate about the active role of EC in the desaturation of fatty acids to linolenic acid in contrast to DA, which probably do not have this ability. Emmanuel (1974) reported direct desaturation of saturated fatty acids to octadecenoic acids in mixed rumen protozoa. Or-Rashid *et al*. (2007) hypothesized that rumen protozoa may have Δ11-desaturese activity, allowing conversion of C18:0 to C18:1 *t*11.

Some authors suggested that protozoa might represent a major pool of unsaturated fatty acids in the rumen (Devillard *et al*. 2006, Yáñez-Ruiz *et al*. 2006, Váradyová *et al*. 2008), although some type of unsaturated fatty acids are of greater content in bacteria than protozoa, i.e., generally odd- and branched-chain fatty acids (Or-Rashid *et al*. 2007). The major sourc-

es of these fatty acids in animal products are of bacterial origin (Keeney *et al*. 1962). Protozoa, however, are rich source of conjugated linoleic acid, especially *c*9*t*11, though the mechanism of so high content it is not clear. According to Or-Rashid *et al*. (2007) protozoa may have also Δ9-desaturese activity that could convert VA to CLA *c*9*t*11. Moreover, protozoa could incorporate CLA *c*9*t*11 from symbiotic bacteria, which isomerase C18:2 *c*9, *c*12 to CLA *c*9*t*11 inside the protozoal cells. According to Jenkins *et al*. (2008) protozoa do not themselves produce CLA and *trans* vaccenic acid by their own metabolism, however, they might be expected to have a significant influence on CLA and *trans* vaccenic acid available to the host animal, that means in milk and meat. The higher concentration of the assayed fatty acids in protozoa is an effect of ingested bacteria. Jenkins *et al*. (2008) suggested that protozoa preferentially incorporate CLA and VA. The study by Or-Rashid *et al*. (2008) puts a new light on the production of CLA and VA by mixed rumen protozoa. They concluded that mixed rumen protozoa are capable of synthesizing CLA from linoleic acid through isomerisation reactions. According to Or-Rashid *et al*. (2008) protozoa are incapable of metabolizing CLA in further stages. They are also incapable of C18:0 desaturation and vaccenic acid biohydrogenation and/or desaturation. Our *in vitro* results indicate that EC and DA do not participate in the production of CLA *c*9*t*11. It seems that the biohydrogenation abilities of the two ciliate species differ from their respective bacterial populations. A major impact of the soluble form of supplemented LA was shown in bacterial fractions of both ciliate cultures. We can speculate about the unavailability of this LA form for ciliates. Other experiments that include fatty-acid analysis of bacteria-free ciliate cells are being conducted using LA absorbed on substrate particulates.

On the basis of the present study, we concluded that experimental rumen ciliates and their associated bacterial populations had different metabolic responses to the tested form and concentration of supplemented LA. Further research is needed evaluating pure particular protozoa species, and also LA may be required in greater concentration.

Acknowledgements. The study was supported by funds from Grant Agency of Ministry of Education of Slovak Republic, the Slovak Academy of Sciences VEGA (2/6174/26 and 2/0009/08) and Grant Agency of Ministry of Science and Higher Education of Poland (2 P06Z 07029). The authors would like to thank A. Nowakowska, M.Sc., M. Kasprowicz, Ph.D., and M. Kubiak, M.Sc. for analytical advice. The authors are also grateful to Dr. P. Siroka for technical assistance.

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Received on 19th June, 2008; revised version on 22nd December, 2008; 15th January, 2009