Prefermented cereals containing fungal gamma-linolenic acid and their effect on rumen metabolism *in vitro*

T. Laho[1], Z. Váradyová[1], K. Mihaliková[1], S. Kišidayová[1], Z. Adamechová[2], M. Čertík[2], D. Jalč[1]

[1]Institute of Animal Physiology, Slovak Academy of Sciences, Košice, Slovak Republic
[2]Department of Biochemical Technology, Faculty of Chemical and Food Technology, Slovak University of Technology, Bratislava, Slovak Republic

ABSTRACT: The application of *Thamnidium elegans* fungal strain CCF 1456 (TE) for effective utilization of various agroindustrial materials creates new perspectives for animal cereal diets enriched with microbial γ-linolenic acid (GLA). Diets consisting of lucerne hay (LH) plus prefermented cereals (wheat bran/spent malt grains, WB+TE or WB+TE enriched with sunflower oil, WB+SO+TE in the first experiment and ground maize grains, GC+TE in the second experiment) were used in the artificial rumen. We examined their effect on the rumen fermentation pattern and lipid metabolism. The diet affected the results of degradability of dry matter, organic matter, neutral detergent fibre and acid detergent fibre of LH+WB diets ($P < 0.05$ and $P < 0.01$). The GLA daily output of prefermented diet substrates LH+WB+TE and LH+WB+SO+TE, or LH+GC+TE was higher compared to the non-prefermented LH+WB or LH+GC, respectively ($P < 0.05$ and $P < 0.01$). Daily outputs of *trans*11 oleic (TVA) of the LH+GC+TE diet were higher *versus* the non-prefermented LH+GC ($P < 0.01$). The biohydrogenation of fatty acids (C18:1 *cis*9 oleic, C18:2 linoleic, C18:3n-3 alpha-linolenic, C18:3n-6 GLA and total FA) of prefermented cereal diets was not influenced. Cereal diets containing microbial GLA might positively enhance GLA daily outputs in the RUSITEC effluent, but they are not effective enough to decrease the biohydrogenation of unsaturated fatty acids.

Keywords: fatty acids; fermentation; fungal strain; gamma-linolenic acid; *in vitro*

A current world trend in the production of diets with supplemented components of polyunsaturated fatty acids (PUFA) has increased the demand for feeds containing γ-linolenic acid (GLA; C18:3n-6) for animal nutrition. Health beneficial PUFA are known as omega-3 (α-linolenic acid, C18:3n-3, ALA; eicosapentaenic acid, C20:5, EPA; docosahexaenoic acid, C22:6, DHA) and omega-6 fatty acids (linoleic acid, C18:2, LA; GLA; arachidonic acid, C20:4, ARA). Omega-3 and omega-6 are not produced by the body and must be obtained through diet or supplementation. Both types of fatty acids (FA) play a role in generating anti-inflammatory compounds in the body and it is recommended that they be consumed approximately at a 4:1 ratio to maximize health benefits. The human body synthesizes GLA from LA by the enzyme Δ-6-desaturase, but humans have a limited activity of Δ-5-desaturase, thus a small fraction of dihomo-γ-linolenic acid (DHGLA) is converted to arachidonic acid (AA). GLA is an intermediate in the conversion of LA to AA and these polyun-

Supported by the Slovak Research and Development Agency (Project No. APVV-No. 0043-07 and project SK-CZ No. 0119-09) and Scientific Grant Agency of the Ministry of Education of the Slovak Republic and Slovak Academy of Sciences – VEGA (Grants No. 2/0001/11 and 2/0021/09).

saturated FA are precursors of a variety of metabolites (prostaglandins, leukotrienes and hydroxyl FA) regulating critical biological functions (Certik and Shimizu, 1999). The primary sources of GLA are plant seed oils (e.g. evening primrose, blackcurrant, borage, or hemp seeds). Limited studies of their effects as diet supplements in calves, cows and growing lambs on rumen degradation characteristics are available (Mustafa et al., 1997; Strzetelski et al., 1998a,b, 1999). Some microorganisms produce oils with significant amounts of GLA as an alternative to agricultural and animal sources (Certik and Shimizu, 1999; Dyal et al., 2005; Sakuradani and Shimizu, 2009). Particularly active in the biosynthesis of GLA are the species of fungi belonging to *Zygomycetes* which have been employed for both submerged and solid state fermentations (Certik, 2008). Of them, the application of *Thamnidium elegans*, *Mortierella isabellina* or *Cunninghamella* species for effective utilization of various agroindustrial materials during solid state fermentations creates new perspectives for e.g. cereal-based pro-ducts enriched with GLA (Certik and Adamechova, 2009). *T. elegans* does not produce any mycotoxins and contains in its biomass neutral lipids esterified by PUFAs of high nutritional and pharmaceutical significance (Vamvakaki et al., 2010). Supplementation of vegetable oils to cereals leads to a rapid increase of PUFA yield in final prefermented products (Certik and Adamechova, 2009). The lipid content of cereal grains in basal diets is variable; however, their fatty acid profile is similar. Cereals are rich in proteins and carbohydrates and are low in fats. Cereal grains are rather low in fats averaging 3.6% of fat. While LA is the major fatty acid of n-6 FA found in grains, ALA is detected in small quantities in cereals. Therefore, cereal-based diets tend to have a high n-6/n-3 ratio and, moreover, are deficient in other essential PUFA (GLA, DHGLA, AA, EPA, DHA).

Previously we tested direct effects of only microbial oil produced by *T. elegans* on rumen metabolism *in vitro* and incomplete biohydrogenation of FA was found (Jalč and Čertík, 2005; Jalč et al., 2005, 2009). Therefore the objective of the present *in vitro* study was to examine the effects of *T. elegans* prefermented cereals such as wheat bran/spent malt grains (LH+WB+TE), wheat bran/spent malt grains enriched with sunflower oil (LH+WB+SO+TE) or ground maize grains (LH+GC+TE) on the rumen metabolism in an artificial rumen.

MATERIAL AND METHODS

Three substrates were used for the preparation of prefermented cereals: wheat bran/spent malt grains (3:1; WB), WB containing 20% of sunflower oil (SO) or ground maize grains (GC), respectively. The fermentation by *Thamnidium elegans* fungal strain CCF 1456 (TE; obtained from the Culture Collection of Fungi, Charles University, Prague, Czech Republic) for the enrichment of cereal substrates with GLA was used. The general incubation cultivation conditions were as described by Certik et al. (2006). Autoclavable microporous polypropylene bags (160 × 270 mm^2) were filled with 10 g of dry substrates (WB or GC), moistened by the addition of 10 ml distilled water, soaked for 2 h at laboratory temperature and sterilized in an autoclave (120 kPa, 120°C, 20 min). The substrates were inoculated with 2 ml of spore suspension (1–2 × 10^6 spores per ml). Each bag was closed with sterile cotton plug and inoculated substrates were arranged to obtain a substrate layer of about 1 cm thickness in the bags. Cultivation was carried out statically at 25°C for 4 days. In order to ensure the homogeneous growth of fungi, prefermented materials were gently agitated once a day during the first two days. To assess reproducibility, triplicate experiments for each substrate were prepared and analysed individually. These prefermented substrates were used as the components of feed ration together with lucerne hay (LH) for the *in vitro* experiments. The nutrient and FA composition of all diet components is in Tables 1 and 2.

The experiments used the rumen simulation technique (RUSITEC). The general incubation period and fermentation equipment included four fermentation vessels (each 850 ml in volume) as described by Jalč et al. (2010). The vessel inoculum was obtained from three ruminally cannulated Slovak merino sheep (age 7 years, mean body weight 45.0 kg) fed 1040 g dry matter (DM) of meadow hay and 260 g DM of crushed barley grains in two equal meals per day. The fermentation inocula (i.e. solid and liquid) were collected through the rumen cannula before morning feeding. The samples were pooled and transferred to the RUSITEC. The solid digesta (80–100 g wet weight) were placed into nylon bags (100 µm pore size) in each fermentation vessel, which were filled to overflowing with strained rumen fluid and McDougall's artificial saliva (1:1; McDougall, 1948). In the first experiment, the four fermentation vessels were supplied with 12 g DM of LH

Table 1. Nutrient composition of diet substrates

	LH	LH+WB	LH+WB+TE	LH+WB+SO	LH+WB+SO+TE	GC	GC+TE
DM (g/kg)	928	940	935	932	948	917	921
N (g/kg DM)	35.5	33.0	35.7	26.0	39.5	19.0	21.0
CP (g/kg DM)	222	206	223	163	184	119	131
NDF (g/kg DM)	515	404	419	378	413	244	185
ADF (g/kg DM)	451	208	243	202	206	84.6	110
Ash (g/kg DM)	67.2	60.0	67.2	51.7	55.9	18.2	29.9
IVDMD (g/kg DM)	577	610	500	561	474	880	806

DM = dry matter, CP = crude protein, NDF = neutral detergent fibre, ADF = acid detergent fibre, IVDMD = *in vitro* dry matter degradability, LH = lucerne hay, WB = wheat bran/spent malt grains (3:1), TE = *Thamnidium elegans*, SO = sunflower oil, GC = ground corn grains

and 3 g DM of WB (i.e. LH+WB; LH+WB+TE; LH+WB+SO and LH+WB+SO+TE, respectively). In the second experiment, the two vessels in duplicates were supplied with 12 g DM of LH and 3 g DM of GC (LH+GC and LH+GC+TE, respectively). Continual infusion of artificial saliva supplemented with microelements (mg/l: $ZnSO_4$ 1.92; $MnSO_4$ 1.02; $CoSO_4$ 0.06) and pH 8.4 was maintained at the rate of 635 ± 11.0 ml through each vessel during the experiments. Each RUSITEC experiment lasted for 11 days. To ensure a steady state within the vessels, adjustment periods were allowed during the first 5 days before the start of sample collections. Measurements were on days 6–11. The experiments were done in duplicate.

Chemical analyses of all diet components (Table 1) and FA composition of LH, WB, SO, WB+TE, WB+SO, WB+SO+TE, GC and GC+TE before

Table 2. Fatty acid composition (g/kg of FA) of diet substrates

Fatty acids	LH	WB	SO	WB+TE	WB+SO	WB+SO+ TE	GC	GC+TE
C14:0 myristic	8.03	4.10	0.8	2.01	1.04	2.01	0.0	3.02
C16:0 palmitic	222	303	61.4	136	89.3	94.2	116	154
C16:1 palmitoleic	17.2	3.12	< 0.005	7.02	1.01	3.02	1.01	6.04
C18:0 stearic	52.2	24.2	31.2	40.1	32.2	30.1	23.2	42.0
C18:1 *cis*9 oleic	108	220	326	294	214	233	348	432
C18:1 *trans*11 oleic	7.3	15.3	10.7	7.03	7.04	7.22	7.21	5.03
C18:2 linoleic	262	376	548	341	634	555	481	282
ALA	153	28.2	10.9	14.2	7.02	5.2	15.2	5.04
GLA	0.0	0.0	0.01	117	0.0	46.2	0.0	49.1
C24:0 lignoceric	97.2	6.01	< 0.005	24.0	3.02	14.2	2.01	9.01
SFA	282	331	103	178	123	126	140	199
MUFA	133	238	337	308	222	243	356	443
PUFA	512	410	559	496	644	621	498	345

ALA = C18:3n-3 α-linolenic acid, GLA = C18:3n-6 γ-linolenic acid, SFA = saturated fatty acids, MUFA = monounsaturated fatty acids, PUFA = polyunsaturated fatty acids, LH = lucerne hay, WB = wheat bran/spent malt grains; SO = sunflower oil, TE = *Thamnidium elegans*, GC = ground corn grains

RUSITEC experiments (Table 2) were in triplicate. The DM of diet substrates was determined by oven drying at 103°C for 16 h. Dried (60°C, 48 h) samples were analysed for neutral detergent fibre (NDF) and acid detergent fibre (ADF) using Fibertec 2010 (Tecator Comp., Höganäs, Sweden) according to Van Soest et al. (1991). 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). *In vitro* dry matter degradability (IVDMD) of diet substrates (Table 1) was as described by Mellenberger et al. (1970).

During the RUSITEC experiments the produced gas was collected into special bags (Tesseraux GmbH, Bürstadt, Germany) and the methane concentrations were analysed in a Perkin-Elmer Clarus 500 gas chromatograph (Perkin-Elmer, Inc., Shelton, USA). The liquid effluent was collected into flasks placed in an ice bath and samples were taken for volatile fatty acids (VFA), ammonia N and FA analyses. Daily production of VFA (total and individual) was analysed by gas chromatography (Cottyn and Boucque, 1968). Ammonia N concentrations were measured by a microdiffussion method (Conway, 1962). FA concentrations in the effluent were determined in lyophilized samples. Lipids were extracted from 500 mg of freeze-dried effluent with chloroform and methanol (2:1) similarly to the feed samples as previously described by Váradyová et al. (2007). The FA methyl ester peaks were identified by authentic standards of a C4–C24 methyl ester mixture (Supelco, Bellefonte, USA). The input of FA (Table 3) was determined from the concentrations of FA in each diet substrate. The analyses of FA were done in 0.5 g of the substrate of each diet and total input was calculated per actual amounts of diet substrate used in fermentation vessels.

The efficiency of microbial protein synthesis (EMS) was calculated from the stoichiometry of rumen fermentation as previously described by Jalč et al. (2010). The growth of the rumen ciliated protozoan population was examined as previously described by Váradyová et al. (2010).

Biohydrogenation (BH) of fatty acids (C18:1, C18:2, C18:3n-3 and C18:3n-6) was calculated from the following equation as the difference between daily intake (daily input) and effluent flow (daily output) as a proportion of daily intake as described by Loor et al. (2003):

Biohydrogenation (%) = 100 – [(C18:1, C18:2 or C18:3 output [mg/day]/total 18-carbon output [mg/day])/(C18:1*cis*9, 18:2n-6 or C18:3 input [mg/day]/total 18-carbon input [mg/day]) × 100]

Statistical analysis used analysis of variance (GraphPad Instat, GraphPad Software Inc., San Diego, USA) as a repeated measures mixed model that represented: (1) four diet groups (i.e. LH+WB, LH+WB+TE, LH+WB+SO and LH+WB+SO+TE) and six time points of measurements (sampling time, experimental days) in two replications for the first experiment; (2) two diet groups in duplicate (i.e. LH+GC and LH+GC+TE) and six time

Table 3. Inputs of fatty acids (mg/day per vessel)

Fatty acids	LH+WB	LH+WB+TE	LH+SO	LH-TE+SO	LH+GC	LH+GC+TE
C14:0 myristic	4.13	3.96	4.04	4.01	3.84	5.04
C16:0 palmitic	146	130	147	122	118	177
C16:1 palmitoleic	7.87	9.09	7.95	8.19	7.57	10.5
C18:0 stearic	26.5	32.3	40.6	30.7	26.8	44.5
C18:1 *cis*9 oleic	82.9	119	168	110	111	272
C18:2 linoleic	175	197	471	263	202	261
ALA	71.1	70.0	70.5	67.9	66.9	69.2
GLA	0.0	34.5	0.0	18.5	0.0	31.6
C24:0 lignoceric	40.2	48.0	43.9	46.0	42.6	42.5

ALA = C18:3n-3 α-linolenic acid, GLA = C18:3n-6 γ-linolenic acid, LH = lucerne hay, WB = wheat bran/spent malt grains, TE = *Thamnidium elegans*, SO = sunflower oil, GC = ground corn grains

Table 4. Effects of diets containing LH with WB or WB containing 20% of SO, respectively, enriched with GLA on fermentation in the RUSITEC effluent

	LH +WB	LH+WB+TE	LH+WB+SO	LH+WB+SO+TE	SEM	Time (T)	Diet (D)	T × D
DMD (g/kg)	571	537	546	515†	12.8	***	*	NS
OMD (g/kg DM)	542	506	522	472††	14.4	***	**	NS
NDFD (g/kg DM)	369	307	352	325	19.6	***	*	NS
ADFD (g/kg DM)	409	405	366	364	18.1	***	*	NS
pH	6.76	6.72	6.74	6.76	0.041	***	NS	***
VFA (mmol/day)	52.9	46.1	50.2	45.4	3.12	***	NS	**
Acetate (mmol/day)	32.4	27.9	30.7	27.2	1.89	***	NS	**
Propionate (mmol/day)	10.7	9.32	10.6	9.21	0.75	***	NS	***
n-Butyrate (mmol/day)	5.26	4.45	4.89	4.31	0.262	***	NS	*
Methane (mmol/day)	5.92	5.03	4.92	4.82	0.39	***	NS	***
Ammonia N (mg/dl)	39.6	44.4†	37.9	40.2	1.08	NS	***	NS
EMS (mg/g)	23.5	25.9††	26.5††	29.9††	0.45	NS	**	NS

DMD = dry matter degradability, OMD = organic matter degradability, NDFD = neutral detergent fibre degradability, ADFD = acid detergent fibre degradability, VFA = volatile fatty acids, EMS = efficiency of microbial protein synthesis, LH = lucerne hay, WB = mixture wheat bran/spent malt grains (3:1), TE = *Thamnidium elegans*, SO = sunflower oil, SEM = standard error of the mean

*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; NS = not significant

$^{\dagger}P < 0.05$; $^{\dagger\dagger}P < 0.01$ differences from LH+WB

points of measurements (sampling time, experimental days) in two replications for the second experiment. Effects included in the model were time (T), diet (D) and interaction between time and diet (T × D). Differences between non-enriched GLA diet (i.e. LH+WB or LH+GC, respectively) and enriched GLA diet (i.e. LH+WB+TE, LH+WB+SO and LH+WB+SO+TE or LH+GC+TE, respectively) were analysed by two-way ANOVA with Bonferroni post-test. Differences between the treatment means were considered to be significant when $P < 0.05$.

RESULTS AND DISCUSSION

Rumen fermentation pattern

Depending on the substrates, GLA amounts in prefermented cereals were WB+TE > GC+TE > WB+SO+TE (117, 49.1, 46.2 g/kg of FA, respectively; Table 2). It should be emphasized that there have not been any available data documenting the testing of cereals enriched with GLA as a supplement to the diet for ruminants yet. As Table 3 shows, the daily input of GLA in the diet substrates (LH mixed with prefermented cereals) was in the range of 18.5 to 34.5 mg/day. In the first experiment, the time (T) and the diet (D) affected the results of DM, OM, NDF and ADF degradability of LH+WB diets ($P < 0.05$, 0.01, and $P < 0.001$; Table 4). Compared to LH+WB diet, the DM and OM degradability of LH+WB+SO+TE diet was lower ($P < 0.05$ and $P < 0.001$). The negative effect of dietary PUFA on fibre digestion and organic matter rumen degradation was also reported by Machmuller et al. (1998) in RUSITEC and by Wachira et al. (2000) in *in vivo* experiments in wethers. The values of pH were influenced by the T ($P < 0.001$). The T × D interaction was effective in the results of pH, total VFA, acetate, propionate, *n*-butyrate and methane ($P < 0.05$, $P < 0.01$ and $P < 0.001$). The concentration of ammonia N was influenced by the D ($P < 0.001$); it ranged from 37.9 to 44.4 mg/dl and in LH+WB+TE diet it was increased compared to LH+WB. The results of EMS of diets enriched with GLA were higher as compared to LH+WB diet ($P < 0.01$). Previously we tested only the effect of purified microbial oil (30 g/kg; w/w) originating from *T. elegans* that was supplemented to the total mixed ration (hay and

barley, 800:200, w/w) in RUSITEC fermentation; the basal parameters of rumen fermentation such as pH, methane, ammonia N as well as DMD and degradability of OM, NDF and ADF were not affected by the diet (Jalč et al., 2005). These findings are consistent with the results of our second experiment described in this paper, where the values of fermentation parameters of LH+GC+TE diet were not affected by the D (Table 5). In addition, the T and the T × D interaction affected the results of pH, total VFA, acetate, propionate, *n*-butyrate and methane ($P < 0.05$, $P < 0.01$ and $P < 0.001$). However, the concentrations of total and individual VFA in enriched cereals were not influenced by the diet. This contradicts the results of Jalč and Čertík (2005) and Jalč et al. (2009), who reported that direct supplementation of microbial oil (30 g per kg; w/w) or oil blends (microbial and fish oil) to the total mixed ration in RUSITEC fermentation reduced a molar proportion of acetate and increased a molar proportion of propionate. Changes in VFA concentrations after the *in vitro* fermentation of the substrate supplemented with sources of GLA (90 g/kg of FA in evening primrose and 230 g/kg of FA in borage seed oil) were described also by Szumacher-Strabel et al. (2009).

Fatty acids and biohydrogenation

Benefits of oils with a high content of GLA (i.e. microbial, evening primrose, borage and black currant) are associated with their potential especially in human medicine (Barre, 2001), however, few studies have been available on effects of GLA on the rumen lipid metabolism. In our first experiment, the T and the D affected the daily outputs of fatty acids (FA) in all LH+WB diets (Table 6). The T × D interaction was effective in FA outputs except PUFA, SFA, MUFA and total lipids ($P < 0.001$). Myristic and pentadecanoic acid daily outputs were lower ($P < 0.05$ and $P < 0.01$) and stearic, *trans* and *cis*9 oleic, *trans*11 oleic (TVA) and CLA daily outputs were higher ($P < 0.05$ and $P < 0.01$) in the RUSITEC effluent of LH+WB+SO diet as compared to LH+WB diet. None of the WB diets enriched with GLA (i.e. LH+WB+TE and LH+WB+SO+TE) was effective in increasing TVA and CLA concentrations in the RUSITEC effluent. It was probably due to the fact that LA content in WB was relatively low (376 g/kg of FA) and CLA is formed from LA. The GLA enriched diets were not effective enough to increase TVA and CLA because GLA does not participate in the formation CLA and TVA. Daily

Table 5. Effects of diets containing LH with GC enriched with GLA on the fermentation pattern in the RUSITEC effluent

	LH+GC	LH+GC+TE	SEM	Time (T)	Diet (D)	T × D
DMD (g/kg)	564	595	18.4	NS	NS	NS
OMD (g/kg DM)	544	579	19.3	NS	NS	NS
NDFD (g/kg DM)	316	316	28.3	NS	NS	NS
ADFD (g/kg DM)	278	320	30.0	NS	NS	*
pH	6.69	6.75	0.038	***	NS	**
VFA (mmol/day)	48.3	41.7	3.42	***	NS	*
Acetate (mmol/day)	28.3	29.5	1.63	***	NS	*
Propionate (mmol/day)	10.0	10.4	0.72	***	NS	*
n-Butyrate (mmol/day)	5.53	6.19	0.272	***	NS	***
Methane (mmol/day)	3.69	4.06	0.279	***	NS	***
Ammonia N (mg/dl)	3.43	3.63	0.178	NS	NS	NS
EMS (mg/g)	25.4	28.6	1.34	NS	NS	NS

DMD = dry matter degradability, OMD = organic matter degradability, NDFD = neutral detergent fibre degradability, ADFD = acid detergent fibre degradability, VFA = volatile fatty acids, EMS = efficiency of microbial protein synthesis, LH = lucerne hay, GC = ground corn grains, TE = *Thamnidium elegans*, SEM = standard error of the mean

*$P < 0.05$; *$P < 0.01$; ***$P < 0.001$; NS = not significant

Table 6. Daily outputs of fatty acids (mg/day per vessel), their isomers and biohydrogenation of diets containing LH with WB or WB containing 20% of SO, respectively, enriched with GLA in the RUSITEC effluent

Fatty acids	LH+WB	LH+WB+ TE	LH+WB+ SO	LH+WB+ SO+TE	SEM	Time (T)	Diet (D)	T × D
C14:0 myristic	6.50	5.28	3.10†	4.72	0.481	***	***	NS
C15:0 pentadecanoic	12.1	9.49	5.85††	8.79	0.931	***	***	NS
C16:0 palmitic	81.3	60.7	51.8	57.6	6.55	***	**	NS
C18:0 stearic	91.0	74.5	143†	94.9	11.9	***	***	NS
C18:1 *trans*9 oleic	0.61	0.46	2.13†	1.02	0.377	***	**	NS
TVA	9.43	9.28	20.0††	12.9	1.51	***	***	NS
C18:1 *cis*9 oleic	2.71	2.38	3.80†	2.86	0.272	***	**	NS
CLA	0.56	0.72	1.41†	0.99	0.185	***	***	NS
C18:2 linoleic	31.3	13.6††	12.4††	19.1††	2.30	***	***	NS
ALA	0.61	0.53	0.70	0.42	0.042	***	*	NS
GLA	0.007	0.340†	0.001	0.420†	0.2611	***	**	NS
Total lipids	236	177	244	204	32.5	***	*	***
SFA	191	150	204	166	20.9	***	*	***
MUFA	12.8	12.1	25.9	16.8	5.69	***	*	***
PUFA	32.5	15.9†	14.0†	20.9	4.61	***	**	NS
Biohydrogenation of FA (%)								
C18:1 *cis*9 oleic	99.7	99.5	99.1	99.5	2.32	NS	NS	NS
C18:2 linoleic	98.4	98.4	99.0	98.6	1.01	NS	NS	NS
ALA	99.9	99.8	99.6	99.9	0.52	NS	NS	NS
Total FA	99.3	99.2	99.2	99.3	0.54	NS	NS	NS

TVA = *trans*11 oleic, CLA = *cis*9 *trans*11 linoleic, ALA = C18:3n-3 α-linolenic acid, GLA = C18:3n-6 γ-linolenic acid, SFA = saturated fatty acids, MUFA = monounsaturated fatty acids, PUFA = polyunsaturated fatty acids, LH = lucerne hay, WB = mixture of wheat bran/spent malt grains (3:1), TE = *Thamnidium elegans*, SO = sunflower oil, SEM = standard error of the mean

*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; NS = not significant

$^{\dagger}P < 0.05$; $^{\dagger\dagger}P < 0.01$ differences from LH+WB

output of linoleic acid of all diets (i.e. LH+WB+TE, LH+WB+SO and LH+WB+SO+TE) was lower ($P < 0.01$) compared to LH+WB. The GLA daily output of LH+WB+TE and LH+WB+SO+TE diets was higher compared to LH+WB. Outputs of PUFA of LH+WB+TE and LH+WB+SO diets were lower than those of LH+WB. Szumacher-Strabel et al. (2009) reported a positive increasing amount of TVA (by almost 20%) in rumen fluid by the addition of evening primrose oil *in vitro.* In addition, direct effects of microbial oil (30 g/kg; w/w) or oil blends (microbial and fish oil) on lipid metabolism in the RUSITEC effluent cause the incomplete BH of unsaturated fatty acids with an increase in TVA concentration (Jalč et al., 2005, 2009). None of the WB diets enriched with GLA (i.e. LH+WB+TE and LH+WB+SO+TE) was effective enough to decrease BH of FA. The daily output of FA from the fermentation of LH+WB+SO diet tends to be high in TVA and CLA outputs. In addition, the SO diet supplementation into the rumen of sheep or RUSITEC fermentation impacted TVA and CLA contents probably from a higher proportion of oleic acid, which can form TVA for the endogenous synthesis of CLA during BH to stearic acid (Váradyová et al., 2008; Jalč et al., 2010).

In the second experiment, the T affected the daily outputs of all fatty acids in LH+GC diets (Table 7). Lower daily outputs of myristic, pentadecanoic, linoleic acid and ALA ($P < 0.05$, $P < 0.01$ and $P < 0.001$) in the RUSITEC effluent of LH+GC+TE diet were accompanied by higher daily outputs of *trans*9 oleic, TVA and GLA ($P < 0.01$) compared to LH+GC diet. It is known that BH reduces the rumen outflow of PUFA and contributes to the accumulation of *cis* and *trans* isomers in ruminant products, including CLA and TVA (Fievez et al., 2007). The BH of FA (C18:1, C18:2, ALA, GLA and total FA) of the LH+GC+TE diet was not influenced. However, the average values of BH in the present experiment in all LH+WB and LH+GC diets were similar (C18:1 99.5%; C18:2 98.6%; C18:3 n-3 99.1%; C18:3n-6 99.0% or C18:1 98.2%; C18:2 99.4%; C18:3n-3 99.5% and C18:3n-6 99.4%, respectively). These findings are consistent with previous results describing almost complete BH (85–100%) for linolenic acid and from 70 to 95% for linoleic acid (Doreau and Ferlay, 1994). BH of GLA in the present experiment, ranked by diet, was as follows: LH+GC+TE > LH+WB+TE > LH+WB+SO+TE (99.81, 98.99 and 97.72%, respectively).

Rumen ciliate protozoan population

The growth of rumen ciliated protozoa in present experiments was very poor in all experimental diets.

Table 7. Daily outputs of fatty acids (mg/day per vessel), their isomers and biohydrogenation of diet containing LH with GC enriched with GLA in the RUSITEC effluent

Fatty acids	LH +GC	LH+GC+TE	SEM	Time (T)	Diet (D)	T × D
C14:0 myristic	6.42	5.30†	0.330	***	**	***
C15:0 pentadecanoic	7.59	6.28†	0.310	***	***	***
C16:0 palmitic	62.2	59.2	2.78	***	NS	NS
C18:0 stearic	111	113	6.8	***	NS	NS
C18:1 *trans*9 oleic	0.59	0.93††	0.053	***	**	NS
TVA	8.46	11.7††	0.621	***	***	***
C18:1 *cis*9 oleic	13.0	14.1	0.98	***	NS	NS
CLA	0.81	0.71	0.078	***	NS	NS
C18:2 linoleic	8.94	4.21††	0.798	***	***	***
ALA	1.72	1.31††	0.077	***	**	NS
GLA	0.003	0.18††	0.008	***	***	NS
Total lipids	221	217	5.3	***	NS	NS
SFA	187	184	1.9	***	NS	NS
MUFA	22.1	26.7	2.38	***	NS	NS
PUFA	11.5	6.41	2.521	***	***	***
Biohydrogenation of FA (%)						
C18:1 *cis*9 oleic	98.1	98.3	2.03	NS	NS	NS
C18:2 linoleic	99.3	99.5	1.95	NS	NS	NS
ALA	99.6	99.4	1.62	NS	NS	NS
Total FA	99.0	99.1	7.22	NS	NS	NS

TVA = *trans*11 oleic, CLA = *cis*9 *trans*11 linoleic, ALA = C18:3n-3 α-linolenic acid, GLA = C18:3n-6 γ-linolenic acid, SFA = saturated fatty acids, MUFA = monounsaturated fatty acids, PUFA = polyunsaturated fatty acids; LH = lucerne hay, GC = ground corn grains, TE = *Thamnidium elegans*, SEM = standard error of the mean

*$P < 0.01$; ***$P < 0.001$; NS = not significant

$^{\dagger}P < 0.05$; $^{\dagger\dagger}P < 0.01$ differences from LH+GC

Only *Dasytricha ruminantium*, *Isotricha* spp., and *Entodinium* spp. were detectable. The ciliate growth was less than 10/ml. These findings are consistent with Kišidayová et al. (2006), who described a decrease in the population of some rumen ciliates after the addition of microbial oil (30 g/kg; w/w) or oil blends (microbial and fish oil) to the rumen fluid fermentation. As the ciliates were not able to grow at experimental diets, we assume that our results of lipid BH in RUSITEC experiments might be ascribed to bacterial activities. The poor growth of ciliates *in vitro* can be ascribed to lucerne saponins which can reduce not only the ciliate growth but also microbial fermentation and synthesis (Lu and Jorgensen, 1987; Lu et al., 1987). Rumen ciliate protozoa are rich in unsaturated FA, especially CLA and TVA, and their FA analysis indicates that the capacity of ciliates to store FA depends on ciliate species and source of dietary lipids (Devillard et al., 2006; Yánez-Ruiz et al., 2006; Cieslak et al., 2009a). In addition, different metabolic responses of rumen ciliates and their associated bacteria to the form and concentration of PUFA sources were suggested previously (Cieslak et al., 2009b).

CONCLUSION

Obviously, it is possible to enhance the concentration of bioactive FA in ruminant-derived food products through animal diet modification only when understanding the microbial ecology of lipolysis and BH and when understanding the relationship between dietary lipid supply and rumen fermentation (Or-Rashid et al., 2009). The diets enriched with microbial GLA were not effective (LH+WB+TE and LH+WB+SO+TE) or they were partially effective (LH+GC+TE) to increase TVA and CLA daily outputs in the RUSITEC effluent. The explanation consists in the fact that GC and GC+TE diet substrates contain other compounds that might elevate the TVA and CLA output during rumen fermentation. All three diets enriched with microbial GLA resulted in higher GLA daily outputs. While the LH+WB+SO diet tends to be high in TVA and CLA concentrations, no effect was observed for the LH+WB+SO+TE diet. None of the diets enriched with microbial GLA was effective enough to decrease BH of C18:1, C18:2 and C18:3. However, more studies are needed to determine the impact of cereals enriched with GLA as diet components which give feed a useful effect above their nutritional value.

Acknowledgement

The authors thank Dr. P. Siroka for gas chromatography analysis and Mrs. V. Venglovská for technical assistance.

REFERENCES

AOAC (1990): Official Methods of Analysis. In: Herlick K. (ed.): Association of Official Analytical Chemists. 15th ed. Arlington, USA, 1230 pp.

Barre D.E. (2001): Potential of evening primrose, borage, blackcurrant and fungal oils in human health – a review. Annals of Nutrition and Metabolism, 45, 45–57.

Certik M. (2008): Biotransformation of oils to value added compounds. In: Hou C.T., Shaw J.F. (eds.): Biocatalysis and Bioenergy. John Wiley & Sons, New York, 571–585.

Certik M., Adamechova Z. (2009): Cereal-based bioproducts containing polyunsaturated fatty acids. Lipid Technology, 21, 250–253.

Certik M., Shimizu S. (1999): Biosynthesis and regulation of microbial polyunsaturated fatty acid production. Journal of Bioscience and Bioengineering, 87, 1–14.

Čertík M., Sláviková L., Masrnová S., Šajbidor J. (2006): Enhancement of nutritional value of cereals with gamma-linolenic acid by fungal solid-state fermentations. Food Technology and Biotechnology, 44, 75–82.

Cieslak A., Miltko R., Belzecki G., Szumacher-Strabel M., Michalowski T. (2009a): Rumen ciliates *Entodinium caudatum*, *Eudiplodinium maggii* and *Diplolastron affine*: a potential reservoir of unsaturated fatty acids for the host. Acta Protozoologica, 48, 335–340.

Cieslak A., Váradyová Z., Kišidayová S., Szumacher-Strabel M. (2009b): The effects of linoleic acid on the fermentation parameters, population density, and fatty-acid profile of two rumen ciliate cultures, *Entodinium caudatum* and *Diplolastron affine*. Acta Protozoologica, 48, 51–61.

Conway E.J. (1962): Microdiffusion Analysis and Volumetric Error. 3rd ed. Crosby-Lockwood, London.

Cottyn B.G., Boucque C.V. (1968): Rapid method for the gas chromatographic determination of volatile fatty acids in rumen fluid. Journal of Agricultural and Food Chemistry, 16, 105–107.

Devillard E., McIntosh F.M., Newbold C.J., Wallace R.J. (2006): Rumen ciliate protozoa contain high concentrations of conjugated linoleic acid and vaccenic acid, yet do not hydrogenate linoleic acid or desaturate stearic acid. British Journal of Nutrition, 96, 697–704.

Doreau M., Ferlay A. (1994): Digestion and utilization of fatty acids by ruminants. Animal Feed Science and Technology, 45, 379–396.

Dyal S.D., Bouzidi L., Narine S.S. (2005): Maximizing the production of γ-linolenic acid in *Mortierella ramanniana* var. *ramanniana* as a function of pH, temperature and carbon source, nitrogen source, metal ions and oil supplementation. Food Research International, 38, 815–829.

Fievez V., Vlaeminck B., Jenkins T., Enjalbert F., Doreau M. (2007): Assessing rumen biohydrogenation and its manipulation *in vivo, in vitro* and *in situ.* European Journal of Lipid Science and Technology, 109, 740–756.

Jalč D., Čertík M. (2005): Effect of microbial oil, monensin and fumarate on rumen fermentation in artificial rumen. Czech Journal of Animal Science, 50, 467–472.

Jalč D., Potkanski A., Szumacher-Strabel M., Cieslak A., Čertík M. (2005): Effect of microbial oil, evening primrose oil and borage oil on rumen fermentation *in vitro*. Veterinarni medicina, 50, 480–486.

Jalč D., Čertík M., Kundríková K., Kubelková P. (2009): Effect of microbial oil and fish oil on rumen fermentation and metabolism of fatty acids in artificial rumen. Czech Journal of Animal Science, 54, 229–237.

Jalč D., Váradyová Z., Lauková A. (2010): Effect of inoculated corn silage enriched with sunflower oil on rumen fermentation and lipid metabolism in an artificial rumen (RUSITEC). Journal of the Science of Food and Agriculture, 90, 78–84.

Kišidayová S., Mihaliková K., Váradyová Z., Potkański A., Szumacher-Strabel M., Cieślak A., Čertík M., Jalč D. (2006): Effect of microbial oil, evening primrose oil, and borage oil on rumen ciliate population in artificial rumen (RUSITEC). Journal of Animal and Feed Sciences, 15, 153–156.

Loor J.J., Hoover W.H., Miller-Webster T.K., Herbein J. H., Polan C.E. (2003): Biohydrogenation of unsaturated fatty acids in continuous culture fermenters during digestion of orchardgrass or red clover with three levels of ground corn supplementation. Journal of Animal Science, 81, 1611–1627.

Lu C.D., Jorgensen N.A. (1987): Alfalfa saponins affect site and extent of nutrient digestion in ruminants. Journal of Nutrition, 117, 919–927.

Lu C.D., Tsai L.S., Schaefer D.M., Jorgensen N.A. (1987): Alteration of fermentation in continuous culture of mixed rumen bacteria by isolated alfalfa saponins. Journal of Dairy Science, 70, 799–805.

Machmuller A., Ossowski D.A., Wanner M., Kreuzer M. (1998): Potential of various fatty feeds to reduce methane release from rumen fermentation *in vitro* (Rusitec). Animal Feed Science and Technology, 71, 117–130.

McDougall E.I. (1948): Studies on ruminant saliva. I. The composition and output of sheep's saliva. Biochemical Journal, 43, 99–109.

Mellenberger R.W., Satter L.D., Millet M.A., Baker A.J. (1970): An *in vitro* technique for estimating digestibility of treated and untreated wood. Journal of Animal Science, 30, 1005–1011.

Mertens D.R. (2002): Gravimetric determination of amylase-treated neutral detergent fibre in feeds with refluxing beakers or crucibles: collaborative study. Journal of the Association of Official Analytical Chemists International, 85, 1217–1240.

Mustafa A.F., McKinnon J.J., Thacker P.A., Christensen D.A. (1997): Effect of borage meal on nutrient digestibility and performance of ruminants and pigs. Animal Feed Science and Technology, 64, 273–285.

Or-Rashid M.M., Wright T.C., McBride B.W. (2009): Microbial fatty acid conversion within the rumen and the subsequent utilization of these fatty acids to improve the healthfulness of ruminant food products. Applied Microbiology and Biotechnology, 84, 1033–1043.

Sakuradani E., Shimizu S. (2009): Single cell oil production by *Mortierella alpine*. Journal of Biotechnology, 144, 31–36.

Strzetelski J.A., Kowalczyk J., Krawczyk K., Stasiniewicz T., Lipiarska E. (1998a): Evening primrose (*Oenothera paradoxa*) oil cake or ground rape seed supplement to diets for dairy cows. Journal of Animal and Feed Sciences, 7, 365–375.

Strzetelski J.A., Kowalczyk J., Niwinska B., Krawczyk K., Maciaszek K. (1998b): A note on rearing calves on diets supplemented with evening primrose (*Oenothera paradoxa*) oil cake. Journal of Animal and Feed Sciences, 7, 377–383.

Strzetelski J.A., Kowalczyk J., Stasiniewicz T., Lipiarska E., Osieglowski S., Bilik K. (1999): Milk yield and composition in dairy cows fed a diet with evening primrose (*Oenothera paradoxa*) full fat seeds or oil cake. Journal of Animal and Feed Sciences, 8, 89–94.

Szumacher-Strabel M., Cieslak A., Nowakowska A. (2009): Effect of oils rich in linoleic acid on *in vitro* rumen fermentation parameters of sheep, goats and dairy cows. Journal of Animal and Feed Sciences, 18, 440–452.

Vamvakaki A.N., Kandarakis I., Kaminarides S., Komaitis M., Papanikolaou S. (2010): Cheese whey as a renewable substrate for microbial lipid and biomass production by *Zygomycetes*. Engineering in Life Sciences, 10, 348–360.

Van Soest P.J., Robertson J.B., Lewis B.A. (1991): Methods for dietary fiber neutral detergent fiber, and non-starch

polysaccharides in relation to animal nutrition. Journal of Dairy Science, 74, 3583–3597.

Váradyová Z., Kišidayová S., Siroka P., Jalč D. (2007): Fatty acid profiles of rumen fluid from sheep fed diets supplemented with various oils and effect on the rumen ciliate population. Czech Journal of Animal Science, 52, 399–406.

Váradyová Z., Kišidayová S., Siroka P., Jalč D. (2008): Comparison of fatty acid composition of bacterial and protozoal fractions in rumen fluid of sheep fed diet supplemented with sunflower, rapeseed and linseed oils. Animal Feed Science and Technology, 144, 44–54.

Váradyová Z., Kišidayová S., Lauková A., Jalč D. (2010): 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. Czech Journal of Animal Science, 55, 105–115.

Wachira A.M., Sinclair L.A., Wilkinson R.K., Hallet K., Enser M., Wood J.D. (2000): Rumen biohydrogenation of n-3 polyunsaturated fatty acids and their effect on microbial efficiency and nutrient digestibility of sheep. Journal of Agricultural Science, 135, 419–428.

Yánez-Ruiz D.R., Scollan N.D., Merry R.J., Newbold C.J. (2006): Contribution of rumen protozoa to duodenal flow of nitrogen, conjugated linoleic acid and vaccenic acid in steers fed silages differing in their water-soluble carbohydrate content. British Journal of Nutrition, 96, 861–869.

Received: 2010–04–14
Accepted after corrections: 2010–12–07

Corresponding Author

Zora Váradyová DVM, PhD., Institute of Animal Physiology, Slovak Academy of Sciences, Šoltésovej 4–6, 040 01 Košice, Slovak Republic
Tel. +421 557 287 841, fax +421 557 287 842, e-mail: varadyz@saske.sk