Effect of microbial oil, monensin and fumarate on rumen fermentation in artificial rumen

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ABSTRACT: The objective of this study was to investigate the effect of microbial oil on rumen fermentation of a diet composed of 60% hay and 40% barley in an artificial rumen (Rusitec). Microbial oil (MO) was produced by the fungus *Thamnidium elegans*. This fungus grew on the wheat bran/spent malt grains (3:1) mixture. The fatty acid composition of microbial oil was as follows: 0.7% $C_{14:0}$, 15.4% $C_{16:0}$, 10.1% $C_{18:0}$, 50.9% $C_{18:1}$, 13.9% $C_{18:2}$ and 8.4% $C_{18:3}$ (GLA, γ-linolenic acid). The effect of monensin MON (66 ppm) and fumarate FUM (6.25 mmol) with and without MO supplementation was also studied. The experiment in Rusitec lasted 11 days. After a stabilization period (5 days), MO was added to fermentation vessel V_2 (6 days), MON to fermentation vessel V_3 (6 days) and FUM to fermentation vessel V_4 (6 days). MO was also added to V_3 and V_4 on the last day together with MON (V_3) and FUM (V_4). The fermentation vessel V_1 served as control (without additives). The results showed that MO reduced ($P < 0.05$) mol% acetate and increased ($P < 0.05$) mol% propionate and *n*-butyrate. Methane production (mmol/day) was reduced numerically (NS). The efficiency of microbial synthesis (EMS) was also reduced numerically and nitrogen incorporated by the microflora (N_M) was reduced significantly in MO supplementation. There were no differences in the rumen fermentation when MO was applied together with MON and FUM compared to the vessel where only MO was applied. No additive effect was observed in the relationship MO-ionophore or MO-FUM. Monensin and fumarate applied separately showed their typical effects on rumen fermentation *in vitro*.

Keywords: microbial oil; monensin; fumarate; rumen fermentation; artificial rumen

Commercial quantities of oils are obtained from animals, plants and oleaginous microorganisms. Oleaginous microorganisms as some species of fungi, marine bacteria, heterophytic and photophytic microalgae, and mosses contain various polyunsaturated fatty acids – PUFA (Certik and Shimizu, 1999). The oils contain PUFA which can be divided into two different categories – n-3 (omega-3) and n-6 (omega-6) fatty acids. Omega-3 fatty acids include α-linolenic acid (ALA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). Omega-6 fatty acids include γ-linolenic acid (GLA), linoleic acid (LA) and arachidonic acid (AA). No metabolic conversion between n-3 and n-6 PUFA is possible (Geay et al., 2001). In this experiment microbial oil produced by oleaginous filamentous fungi was used. It is known that dietary lipids undergo two important transformations in the rumen of ruminants. The initial step is hydrolysis of the ester linkages catalyzed by microbial lipases. The second step is hydrogenation of unsaturated fatty acids by ruminal bacteria (Bauman et al., 1999) characterized by the production of *trans* fatty acids. Several factors influence the rate and extent of ruminal biohydrogenation of PUFA and thus, *trans* vaccenic acid (TVA) and conjugated linoleic acid production (CLA). For example, to remove reductants for biohydrogenation of CLA it is possible to use alternative electron acceptors as ionophores and organic acids as fumarate (Newbold et al., 2001). No information about the effect of microbial oil supplementation to the ruminant diet on rumen

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fermentation *in vitro* was found. Neither were any data presented about the effect of microbial oil on rumen fermentation induced by different electron acceptors – fumarate and monensin. Therefore the aim of this work was to study: (a) the effect of microbial oil on rumen fermentation of a diet composed of 60% hay and 40% barley; (b) the effect of microbial oil on rumen fermentation induced by fumarate and monensin in an artificial rumen. This work will be supplemented (in the next paper) with the results showing the effect of microbial oil, monensin and fumarate supplementation on biohydrogenation of polyunsaturated fatty acids and *trans* fatty acid (CLA, TVA) production in a fermentation fluid in a semicontinuous fermenter (Rusitec).

MATERIAL AND METHODS

In vitro fermentation system

The experiment was carried out using a continuous fermenter (Rusitec) described by Czerkawski and Breckenridge (1977). The fermenter was equipped with four vessels (V_1, V_2, V_3, V_4), each 850 ml in volume. The general incubation period was described by Czerkawski and Breckenridge (1977). The vessel inoculum was obtained from three ruminally cannulated Slovak Merino sheep (mean body weight 44.2 ± 2.1 kg) that were fed 780 g of dry matter (DM) of fresh lucerne and 520 g DM of crushed barley in two equal meals. The chemical composition of lucerne and barley was as follows: DM – 28.96 (88.91); nitrogen – 1.57 (2.18); ash – 3.26 (3.67); neutral detergent fibre (NDF) – 21.37 (26.10); acid detergent fibre (ADF) – 14.23 (6.75); hemicellulose – 7.12 (19.35); cellulose – 13.39 (5.37); lignin – 0.83 (1.38) as % of original DM. Fermentation inocula (solid and liquid) were collected through the rumen cannula immediately before the morning feeding and transferred into an artificial rumen. The solid digesta (80 g wet weight) were placed into nylon bags (100 μm pore size) in each fermentation vessel. The vessels were filled to overflowing with strained rumen fluid and artificial saliva (1:1) (McDougall, 1948). The vessels were supplied with the diet containing 9.0 g DM of fresh lucerne and 6.0 g DM of barley at daily intervals including the first day of the experiment. The fermentation vessel V_2 received also 5% (wt/wt) addition of microbial oil (6 days); V_3 received 1 mg (66 ppm) monensin (6 days) + 5% (wt/wt) supplementation of microbial oil – MO (the last day), V_4 received 1 g (6.25 mmol) of fumarate (6 days) + 5% (wt/wt) MO supplementation (the last day). The fermentation vessel V_1 served as control. A continual infusion of artificial saliva (pH 8.4) at the rate 568–652 ml/day was maintained through each vessel during the experiment.

Additives

Microbial oil was obtained from the Department of Biochemical Technology, Faculty of Chemical and Food Technology, Slovak Technical University, Bratislava, Slovak Republic. Microbial oil was composed of the following fatty acids in %: $C_{14:0}$ – 0.7, $C_{16:0}$ – 15.4, $C_{18:0}$ – 10.1, $C_{18:1}$ – 50.9, $C_{18:2}$ – 13.9, $C_{18:3}$ (GLA) – 8.4.

Monensin, sodium salt (1 mg, 66 ppm – 15 g DM of feed) dissolved in methanol, was added as a single dose to the artificial saliva solution. Stock solution was prepared by dissolving 14.8 mg monensin in 10 ml methanol and 1ml solution was added to artificial saliva (Ø flow 600 ml).

Fumarate (fumaric acid, disodium salt; 1 g) was applied directly into feed. The amount of fumarate corresponds to the concentration 6.25 mmol (6.25 × 160.04 mol weight). Fumarate and monensin were obtained from Sigma Chemical Company, St. Louis, MO, USA.

Measurements and chemical analyses

The duration of the experiment in Rusitec was 11 days. To ensure a steady state within the vessels a 5-day adaptation period was followed by a 6-day collection period. On days 6–10 and 6, 12 and 24 hours of the last day the following samples were collected. The produced gas was collected into special bags and the volumes of gas were measured with gas-meter and methane concentrations were analysed using a gas chromatograph (Perkin-Elmer 8500) as reported by Czerkawski and Clapperton (1968). The liquid effluent was collected in flasks placed into ice bath and samples were taken for volatile fatty acid (VFA) and ammonia nitrogen (NH_3-N) analyses. Daily productions of VFA were analysed by the gas chromatography procedure (Cottyn and Boucque, 1968) using crotonic acid as an internal standard in the gas chromatograph.

Ammonia nitrogen concentration was measured by a microdiffusion method (Conway, 1962). Dry matter, ash and nitrogen were determined according to the methods of the Association of Official Analytical Chemists (AOAC, 1980). Nitrogen (N) incorporated by the microflora (N_M) was estimated as the difference between total nitrogen N_T (nitrogen in the effluent + bag feed residue) and NH_3-N ($N_M = N_T - NH_3$-N) in mg/g (Alves de Oliveira et al., 1997). The other fermentation variables – fermentation efficiency (Orskov et al., 1968), organic matter fermented OMF (Demeyer and Van Nevel, 1979), production, utilization and recovery of metabolic hydrogen (Demeyer, 1991) were calculated according to the stoichiometry of rumen fermentation.

Statistical analysis

Means of results from treatments were compared by one-way analysis of variance (ANOVA). Treatment means were statistically compared by Tukey-Kramer multiple comparison test. The tables show the group means and the standard error of the mean. Probability values of $P < 0.05$ were considered as significant.

RESULTS AND DISCUSSION

The fermentation of the studied diet was carried out at pH 6.87–7.24 and was similar in all fermentation vessels (Table 1). Rumen degradation of dry matter after 48 h of incubation in the fermentation fluid was not affected by the additives (Table 2). The apparent DM digestibility was not significantly different when cows received a concentrate/forage diet (65:35%) supplemented with 5% sunflower (SO), 5% linseed (LO) or 2.5% fish oil (FO) (Loor et al., 2003) or when sheep were fed the diet supplemented with 6% linseed, 6% fish oil or 6% linfish (linseed + fish oil; Wachira et al., 2000). However, fumarate (6.25 mmol) increased (about

Table 1. The effect of microbial oil on pH, gas production, NH_3-N concentration and stoichiometry of rumen fermentation of the diet containing monensin and fumarate in Rusitec

	Control[a] V_1	MO[b] V_2	MON[c] V_3	MON + MO[d] V_3	FUM[e] V_4	FUM + MO[f] V_4	Pooled ± SEM
pH	7.04	7.09	6.96	6.87	7.14	7.24[d]	0.05
NH_3-N (mg/100ml)	40.6[f,d]	39.22	44.31[b]	36.71[c]	39.90	35.99[c]	0.82
N_2 utilization (%)	79.37	80.90	77.82	80.16	75.06	79.48	1.56
Total gas production (l/day)	4.28	3.98	4.32	4.47	4.04	4.38	0.24
E (%)	75.69[b]	77.29[d]	77.31[a]	78.45[c,a]	77.66[a]	76.52[a]	0.28
H_2 production (mmol/day)	121.36[e]	111.44	125.24[e]	114.24	86.09	121.49[e]	5.51
H_2 utilization (mmol/day)	61.96[b]	79.87	87.60[a]	80.21	86.09[a]	80.34	3.48
H_2 recovery (%)	50.91	71.68[a]	70.22[a]	70.68[a]	71.32[a]	66.15[a]	1.83
OMF (g/day)	5.47	5.11	5.75	5.37	5.59	5.66	0.27
N_M (mg/day)	168.35	140.21[a]	148.94	170.99	163.92	173.56	5.86
EMS = N_M/OMF (mg/g)	30.77	27.43	25.90	31.84	29.32	30.66	1.07

E – energetic efficiency of VFA; OMF – organic matter fermented; N_M – nitrogen incorporated by microflora; EMS – efficiency of microbial synthesis; MO – microbial oil, 5% wt/wt; MON – monensin, 66 ppm; FUM – fumaric acid, disodium salt, 6.25 mmol; ± SEM – standard error mean; n = 4–8; values in a row with different superscript letters (a, b, c, d, e, f) differ at $P < 0.05$

6.3%) IVDMD after its addition to the diet containing hay, barley, molasses and fish meal (50:30:10:9%; López et al., 1999). Monensin (2, 10, 50 mg/day) reduced IVDMD when it was added to the hay-barley (70:30%) diet in Rusitec (Wallace et al., 1981). Total VFA, acetate, propionate and *n*-butyrate production (mmol/day) was not influenced by the additives (Table 1). But molar proportions (mol%) of acetate were significantly decreased by all additives. Molar proportions of propionate were increased ($P < 0.05$) by monensin (+3.1%) and microbial oil after monensin application (+7.8%), or numerically (not significantly NS) increased by MO, FUM, FUM + MON (Table 1). The molar proportion of butyrate was increased ($P < 0.05$) by microbial oil supplementation (+2.5%) only compared to the control vessel. The calculation of the data from acetate to propionate production showed a significant decrease in the A/P ratio in all supplemented diets. Most researches also found a reduction in acetate production due to monensin (Wallace et al., 1981), due to oils (Doreau et al., 1993; Jalč et al., 2002) or higher acetate production due to fumarate – 4, 8, 12 mmol (Callaway and Martin, 1996). Usually, propionate production was increased due to monensin (Nagaraja, 1995), fumarate (López et al., 1999) or by oils as canola oil (Wettstein et al., 2000), fish oil (Wachira et al., 2000), linseed, sunflower, rapeseed RO (Jalč et al., 2002) and soybean oils (Jenkins et al., 2003). Our previous (unpublished) results also showed the increase of propionate when monensin (66 ppm) was used individually or in combination with linseed, sunflower and fish oils (5% wt/wt) at the fermentation of the diet composed of 60% fresh lucerne and 40% barley in Rusitec. Total gas production and ammonia nitrogen concentration were not affected by the used additives. Nitrogen utilization (%) calculated from nitrogen contents in feed (input) and in feed residues and effluent (output) was not affected by the used supplements either (Table 2). Compared to the unsupplemented control vessel (V_1), methane production was decreased numerically (NS) (about 16.4%) due to the microbial oil supplementation in V_2. Our previous

Table 2. The effect of microbial oil on rumen fermentation of the diet containing monensin and fumarate in Rusitec

	Control[a] V_1	MO[b] V_2	MON[c] V_3	MON + MO[d] V_3	FUM[e] V_4	FUM + MO[f] V_4	Pooled ± SEM
IVDMD (%)	75.53	67.99	74.78	75.77	71.05	73.48	2.14
VFA production (mmol/day)	58.95	53.37	61.52	59.26	59.73	58.34	2.99
Acetic acid (mmol/day)	31.66	25.19	30.69	29.72	29.68	29.46	1.66
Propionic acid (mmol/day)	12.24	12.09	14.79	16.88	14.34	12.68	0.97
N-butyric acid (mmol/day)	9.27[d]	9.70[d]	9.59[d]	7.15	9.47[d]	9.86[d]	0.38
Acetic acid (mol%)	53.97[b]	47.23[c]	49.87[a]	50.08[a,b]	49.60[a,b]	50.34[a,b]	0.43
Propionic acid (mol%)	20.74[c]	22.52[a]	23.84[d]	28.57[a]	24.03[d]	21.78[d]	0.76
N-butyric acid (mol%)	15.75[b,d]	18.25 [d]	15.71	12.06[c]	15.90[d]	16.89[d]	0.57
A/P ratio	2.59	2.11[a]	2.11[a]	1.76[a]	2.06[a]	2.32	0.07
Methane (mmol/day)	4.08	3.41	4.01	3.78	4.01	3.13	0.07

IVDMD – *in vitro* dry matter digestibility; MO – microbial oil, 5% wt/wt; MON – monensin, 66 ppm; FUM – fumaric acid, disodium salt, 6.25 mmol; ± SEM – standard error mean; $n = 4–8$; values in a row with different superscript letters (a, b, c, d, e, f) differ at $P < 0.05$

studies (Jalč et al., 2002) showed that the other oils – RO, SO, LO also numerically decreased CH_4 production (about 30%, 33% and 30%, respectively) *in vitro*. Monensin and fumarate decreased CH_4 production slightly (about 1.7%, NS) and microbial oil addition to fermentation vessels induced by monensin and fumarate (about 7.3 and 23.3%, NS) decreased it strongly (Table 2). Thus microbial oil interfered with rumen fermentation and caused a certain drop in methane production similar to the effect of ionophores and organic acids such as fumarate (Nagaraja, 1995; López et al., 1999). The other parameters of rumen fermentation (Table 1) were calculated from the stoichiometric relationship in the rumen. The fermentation efficiency (E) and recovery of metabolic H_2 were increased significantly in all supplemented diets (Table 1). Several studies examined the effect of oils on microbial protein synthesis and showed the beneficial effect of PUFA in oils on microbial proteosynthesis (Broudiscou et al., 1994) as well as the negative effect (Czerkawski et al., 1975). The supplementation of microbial oil to the fresh lucerne-barley (60:40%) diet showed that: (a) OMF was not affected by MO; (aa) the amount of microbial nitrogen was reduced significantly; (aaa) EMS was reduced by MO numerically. The other additives (monensin and fumarate) reduced N_M and EMS numerically. Microbial oil applied together with monensin or fumarate increased N_M and EMS slightly (NS) in comparison with the control. The other authors reported microbial protein synthesis reduced by LO and FO supplementation (60 g/kg) or by RO (40 and 80 g/kg) supplementation (Wachira et al., 2000). Our previous (unpublished) results showed that monensin together with the oils SO, LO, FO increased microbial protein synthesis slightly (NS).

In conclusion, it can be stated that microbial oil reduced ($P < 0.05$) mol% acetate and increased ($P < 0.05$) mol% propionate and n-butyrate. Methane production and efficiency of microbial protein synthesis were reduced numerically and nitrogen incorporated by the microflora (N_M) was reduced significantly by microbial oil supplementation. The rumen fermentation was not changed when microbial oil was applied together with monensin and fumarate. No additive effect was observed in the relationship microbial oil-ionophore or microbial oil-fumarate. Monensin and fumarate applied individually showed their typical effects on rumen fermentation *in vitro*. In future, in the next paper, we will present the results describing the effect of microbial oil, monensin and fumarate on rumen biohydrogenation of polyunsaturated fatty acids, and on the production of conjugated linoleic acid and trans-vaccenic acid in an artificial rumen.

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