Fermentation pattern of the rumen and hindgut inocula of sheep grazing in an area polluted from the non-ferrous metal industry

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ABSTRACT: *In vitro* study of the rumen fluid (RF) and hindgut content (HC) fermentation by microbiota taken from sheep grazing in an area atmospherically polluted from the non-ferrous metal industry was conducted and compared with controls from an uncontaminated area (UA). The experimental sheep were exposed to the prolonged intake of heavy metals by grazing in the contaminated area (CA) for one year. Soil and grass from that area and the rumen content of sheep were analyzed for heavy metal levels. Based on the levels of mercury (4.752 mg/kg), copper (232.9 mg/kg), cadmium (1.167 mg/kg), lead (92.509 mg/kg) and arsenic (74.59 mg/kg) the soil was categorized as profusely contaminated. Meadow hay (MH) from UA was used as a tested substrate of fermentation activity; it was incubated with buffered RF and HC inocula from CA and UA for 24 h. The gas volume in CA was significantly decreased by 50 and 36% in RF and HC, respectively. The methane production in CA was significantly decreased by 77 and 71% in RF and HC, respectively. The significantly decreased values of the fermentation parameters in CA in comparison with UA were accompanied by the reduced ($P < 0.01$) total concentration of rumen ciliate protozoa.

Keywords: heavy metals; *in vitro* fermentation; rumen fluid; hindgut content; volatile fatty acids; rumen ciliates

Ruminants may be exposed to toxic concentrations of heavy metals and other trace elements by consuming contaminated forage in pastures. Emissions from industrial areas appear to be a powerful source of heavy metals in plants in specific regions. The ecological aspects of heavy metal pollution in selected agglomerations of Eastern Slovakia and their influences on the course of pasture helminthoses were studied previously (Borošková et al., 1994; Krupicer et al., 1996). The harmful effects of heavy metals on animal health, production as well as anaerobic digestion depend on the kind of the element and concentration or dose of free metals (Mueller and Steiner, 1992). The elements consumed by ruminants may be inhibitory to both the fermentative activity and the growth of microorganisms present in the rumen (Forsberg, 1978). Reduced microbial population or the activity of a particular microorganism may result either from the indirect effect of heavy metals on plant growth and nutrient availability in the soil or from the interaction with other organisms in addition to direct toxicity. The aim of this study was to examine the influence of the rumen fluid and hindgut content inocula from sheep grazing in a non-ferrous metal industry contaminated area on fermentation patterns and activity of rumen ciliates incubated 24 h *in vitro*.

MATERIAL AND METHODS

The rumen fluid (RF) and hindgut content (HC) inocula used in the present study were obtained

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from slaughtered sheep. Six experimental sheep (Merino) were grazed in the area of Kaľava village (Eastern Slovakia) and exposed to prolonged ingestion of heavy metals in the pasture throughout one year. The Kaľava area is contaminated by atmospheric pollution from the non-ferrous metal industry at Krompachy (Eastern Slovakia). During the winter time the sheep were housed in pens and fed meadow hay obtained from the same area. Six control sheep were grazed in an uncontaminated area. Samples of RF and HC were transferred to the laboratory in a water bath preheated to 39 ± 0.5°C, squeezed through four gauze layers, gassed with CO_2 and mixed with McDougall's buffer (McDougall, 1948) at a ratio of 1:2. The 50ml glass gas-tight syringes (Sigma, St. Louis, MO, USA) were used as fermentation vessels. 35 ml of RF or HC inoculum was added with automatic pump to each syringe containing 0.25 g of substrate. Meadow hay from the uncontaminated area (MH) was used as substrate for fermentation. MH was ground through a 0.15–0.4 mm screen, bulked, and stored in a sealed plastic container until required. Six replicate glass syringes were used for the experimental groups with RF (inoculum + buffer + substrate) and six were also used for the controls with RF (inoculum + buffer, no substrate). Six replicate glass syringes were also used for experimental groups with HC (inoculum + buffer + substrate) and six were also used for the controls with HC (inoculum + buffer, no substrate). The glass syringes were then placed on a stand in the incubator for 24-h incubation. During the incubation, the temperature in the incubator was maintained at 39 ± 0.5°C. The concentration of ciliate protozoa was counted microscopically according to Coleman (1978). The samples were collected at the end of fermentation experiments and fixed with 8% formaldehyde solution (1:1). Ciliate genera and species were identified according to Dogiel (1927) and Ogimoto and Imai (1981). The concentration of *Entodinium* spp., *Dasytricha ruminantium, Isotricha* spp., *Ophryoscolex caudatus tricoronatus* and the total number of ciliates were counted. Gas production was measured by a gas-tight syringe method (Váradyová et al., 1998, 2005). Gas from each glass syringe was collected in a 2 ml glass gas-tight syringe (2 ml) at the end of incubation and immediately analyzed for methane concentrations by gas chromatography (Perkin-Elmer 8500). The percentage of methane was expressed per 1 ml gas volume produced. Before and after incubation, the concentrations of volatile fatty acids (VFA) in the inoculum were determined by gas chromatography (Cottyn and Boucque, 1968) using crotonic acid as the internal standard and a Perkin-Elmer 8 500 gas chromatograph. *In vitro* dry matter degradability (IVDMD) was estimated from the difference in the substrate weight prior to and after incubation. The contents of the syringes were transferred into a tube and centrifuged at 3 500 g for 10 min. The residues were washed twice with distilled water, centrifuged and dried to constant weight at 105°C (Mellenberger et al., 1970). The mercury (Hg), copper (Cu), cadmium (Cd), lead (Pb) and arsenic (As) levels in the soil, grass and rumen content were determined by flame atomic absorption spectrometry (AAS). Hg was analyzed on an AMA 254 Hg-analyzer, Cu was analyzed on an AAS UNICAM 939 flame analyzer, Cd, Pb and As were analyzed in a graphite cuvette on AAS UNICAM 939 QZ. For all elements, standards were prepared from commercially available standard solutions for AAS. The samples were mineralized prior to analysis by a mixture of 20 ml HNO_3 and HCl (3:1). Mineralizates were evaporated to volumes of about 5 ml and diluted with demineralized water to final volumes of 25 ml (Resolution of the Ministry of Agriculture of the Slovak Republic, 2004). Gas production, methane, VFA and IVDMD were analyzed by analysis of variance (Graph Pad InStat, Graph Pad Software, Inc. San Diego, USA).

RESULTS

The heavy metal contents in soil, grass and rumen content are shown in Table 1. Of the heavy metals Cu was present at the highest abundance followed by Pb and As. The highest levels of Cu, Cd and As were present in the soil > grass and > in the rumen content. Hg and Pb were most abundant in the soil > rumen content and > grass. Based on the Hg, Cu, Cd, Pb and As content the soil was categorized into profusely contaminated up to contaminated soil (Table 1). In spite of this the contamination of grass was below the toxic limits, except the content of Cd. According to the categorization of pollution (Bulletin of the Ministry of Agriculture of the Slovak Republic, 1994) A, A_1 are limits for dangerous soils (A for the total content of the element, A_1 for the content of the element in 2M HNO_3 and 2M HCl, respectively). The content of at least one of the dangerous elements exceeds limits A, A_1 up to limit B. Soils of B category are

Table 1. Contents of the main heavy metals (mg/kg DM) in soil, grass and rumen content of sheep grazing in the area polluted from the non-ferrous metal industry (DM, dry matter)

Metal	Rumen content sample	Grass		Soil				
		sample	limit	sample	limits			
					A	A_1	B	C
Mercury	0.019 ± 0.4	0.005 ± 0.6	0.1	4.752 ± 1.2	0.3	–	2	10
Copper	5.386 ± 1.9	7.023 ± 1.8	25.0	232.9 ± 4.1	36	20	100	500
Cadmium	0.384 ± 0.7	0.481 ± 0.7	0.3	1.167 ± 0.6	0.8	0.3	5	20
Lead	4.138 ± 1.0	0.552 ± 0.6	5.0	92.509 ± 3.3	85	30	150	600
Arsenic	0.013 ± 0.3	0.487 ± 0.5	0.62	74.59 ± 2.9	29	5	30	50

all values are means ± SEM (n = 3)

A, A_1 – dangerous soil, B – contaminated soil, C – profusely contaminated soil

categorization of pollution (Resolution of the Ministry of Agriculture of the Slovak Republic, 1994)

contaminated soils, the content of at least one of the dangerous elements exceeds limits from B to limit C. C soils are profusely contaminated soils, the content of at least one of the dangerous elements exceeds the limit C. The legislation determines the decontamination and strict control of C soils. According to the categorization of pollution the heavy metal contents in soil, grass and rumen content from the uncontaminated area were below the toxic limits. The effect of inoculum and area on gas volume, methane, IVDMD and total VFA is shown in Table 2. Gas volume, methane, IVDMD, and total VFA of both fermentations (meadow hay, control) were higher in the uncontaminated area compared with the contaminated area. There was no effect of inocula on methane and IVDMD. The mol% values for acetate and *n*-butyrate were higher in the uncontaminated area (Table 3). The mol%

Table 2. Gas volume, methane, *in vitro* dry matter degradability (IVDMD) and total volatile fatty acids (VFA) of meadow hay (MH) incubated 24 h *in vitro* with rumen fluid (RF) and hindgut content (HC) inocula from sheep grazing on a contaminated (CA) and uncontaminated (UA) area

Substrate (S)	Inoculum (I)	Area (A)	Gas volume (ml/g DM)	Methane (10^2 ml/ml)	IVDMD (%)	Total VFA (mM)
MH	RF	CA	88.0	1.4	49.4	50.4
	RF	UA	175.2	6.1	59.5	80.5
Control	RF	CA	39.6	1.3	–	40.6
	RF	UA	43.6	2.4	–	62.7
MH	HC	CA	86.4	1.4	35.6	53.3
	HC	UC	136.8	4.9	44.6	52.2
Control	HC	CA	34.0	1.4	–	47.9
	HC	UC	39.2	2.0	–	44.1
SEM			0.8	0.2	0.9	0.8
Significance		I	***	ns	ns	***
		A	***	***	***	***
		I × A	**	**	ns	***

all values are means; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; ns = not significant

Table 3. Acetate, propionate, n-butyrate of meadow hay (MH) incubated 24 h *in vitro* with rumen fluid (RF) and hindgut content (HC) inocula from sheep grazing in a contaminated area (CA) and uncontaminated area (UA)

Substrate (S)	Inoculum (I)	Area (A)	Acetate (mol%)	Propionate (mol%)	*n*-Butyrate (mol%)	A:P ratio
MH	RF	CA	61.9	22.9	7.8	2.7
	RF	UA	71.9	17.5	7.9	4.1
Control	RF	CA	58.8	19.0	9.2	3.1
	RF	UA	70.4	17.2	8.7	4.1
MH	HC	CA	69.5	19.4	4.6	3.7
	HC	UC	71.1	18.7	5.9	3.8
Control	HC	CA	69.3	20.3	4.4	3.5
	HC	UC	70.7	16.2	5.5	4.3
SEM			0.9	0.3	0.4	0.5
Significance		I	***	*	***	ns
		A	***	***	ns	*
		I × A	***	***	***	ns

all values are means; *P < 0.05; **P < 0.01; ***P < 0.001; ns = not significant

values for propionate were lower in the contaminated area. There were significant effects of the area on mol% of iso-butyrate, valerate and iso-valerate (Table 4). No caproic acid was detectable at fermentation in the uncontaminated area. A significant decrease was determined in the total rumen ciliate and *Entodinium* spp. population in samples from the contaminated area (Figure 1). Significant

Table 4. Iso-butyrate, valerate, iso-valerate and caproate of meadow hay (MH) incubated 24 h *in vitro* with rumen fluid (RF) and hindgut content (HC) inocula from sheep grazing in a contaminated area (CA) and uncontaminated area (UA)

Substrate (S)	Inocula (I)	Area (A)	Iso-butyrate (mol%)	Valerate (mol%)	Iso-valerate (mol%)	Caproate (mol%)
MH	RF	CA	2.3	1.9	3.2	0.1
	RF	UA	1.1	1.0	1.5	–
Control	RF	CA	1.3	1.3	6.4	0.1
	RF	UA	1.5	1.1	1.9	–
MH	HC	CA	1.6	1.7	1.7	0.02
	HC	UC	1.4	1.6	1.4	–
Control	HC	CA	2.1	1.8	2.2	0.04
	HC	UC	2.5	3.3	2.0	–
SEM			0.2	0.1	0.3	0.1
Significance		I	**	ns	ns	ns
		A	***	*	**	ns
		I × A	***	ns	ns	ns

all values are means; *P < 0.05; **P < 0.01; ***P < 0.001; ns = not significant

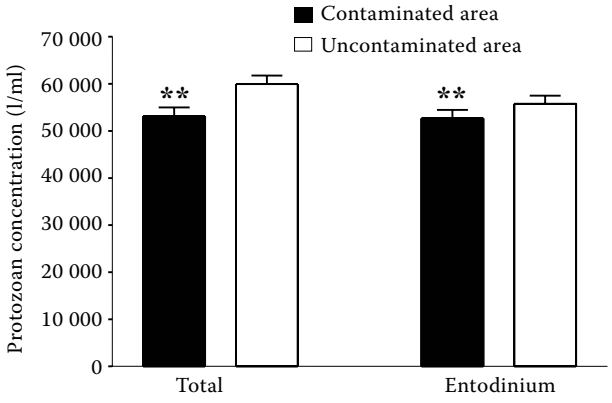

Figure 1. Total protozoan population (Total) and *Entodinium* spp. (Entodinium) in a contaminated and uncontaminated area after 24 h fermentation *in vitro*

$^{**}P < 0.01$

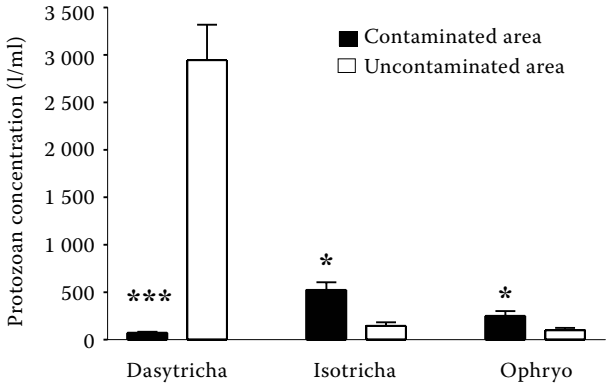

Figure 2. *Dasytricha ruminantium* (Dasytricha), *Isotricha* spp. (Isotricha) and *Ophryoscolex c. tricoronatus* (Ophryo) in a contaminated and uncontaminated area after 24 h fermentation *in vitro*

$^{*}P < 0.05$; $^{***}P < 0.001$

differences between contaminated and uncontaminated samples were observed as to the concentration of *Dasytricha ruminantium, Isotricha* and *Ophryoscolex c. tricoronatus* (Figure 2).

DISCUSSION

The soil in the examined area proved to be profusely contaminated. The contamination is associated with prolonged atmospheric pollution from the nonferrous metal industry. A previous study (Krupicer, 1995) showed that the Hg concentration in grass was influenced by the distance of emission source with the mean Hg concentration of 4.298 mg/kg in the area lying 3 km from the emission source. However, in the present study the toxicological limits were not exceeded in grass (0.005 mg/kg). It is clear that unfavourable effects on the animal health depend on the kind of element and its dose as well as on animal utility orientation. Microbial digestion of feed in the rumen involves a sequential microbial attack culminating in the formation of fermentation products (especially volatile fatty acids) that can be utilized by the host animal. However, digestion of food particles depends not only on the activity of the microorganisms but also on the health of the animals. Regarding our study the main finding was a much lower production of methane, total gas, total VFA and acetate in the rumen fluid and hindgut content inocula of sheep grazed in the contaminated area compared to the uncontaminated area. It is known from the literature data (Chiu-Yue Lin, 1992) that the archea responsible for methanogenesis are generally considered to be more sensitive to environmental conditions such as toxicant concentrations than other intestine microbes. In fermentation from contaminated areas obviously depressed the gas and methane production, production of VFA, altered the fermentation pattern and induced accumulation of hydrogen to produce new fermentation end products (decrease or increase of individual VFA production). Probably as a consequence of inhibition the microbial activity and the methanogenic population appeared not to be fully able to consume hydrogen produced in bacterial and protozoan communities. However, it is clear that when using an *in vitro* technique, the variables such as diet of the donor animals, adaptation of the microbial population, time of collection of RF or HC after feeding and degree of anaerobiosis may affect fermentation parameters. The effect of Cu and Cd administration on protozoan populations was described in several studies (Zeleňák et al., 1992; Sviatko and Zeleňák, 1993; Jalč et al., 1994; Kišidayová et al., 2000a,b), and protozoan growth seems to be more sensitive to excessive levels of heavy metals than bacterial growth. Cu was the most abundant heavy metal present in soil, grass and rumen content of sheep from the contaminated area. It is one of the most toxic heavy metals *in vitro* may have a depressive effect on microbial digestion *in vivo* when fed at excess of about 100 mg/kg DM (dry matter), since its solubility in the rumen was

shown to rise with increased dietary levels (Wetzel and Menke, 1978). The data also show the overall high toxicity of Cu at quite low levels. According to Beswick et al. (1976) the toxicity of Cu in an anaerobic culture is apparently due to the cuprous (Cu^{1+}) ion. However, it is known that different species of rumen bacteria do not reveal the same sensitivity to Cu (Forsberg, 1978). According to Ivan et al. (1986) the rumen ciliate protozoa can alleviate the chronic Cu toxicity in ruminants by the formation of CuS in consequence of sulphur amino acid degradation. However, this ability also depends on the biomass of protozoan population (Kišidayová et al., 2000a). From the aspect of toxicity sheep are more sensitive to Cu toxicity than other farm animals. It is known that e.g. goats are more resistant to Cu toxicity than sheep (Adams et al., 1977; Solaiman et al., 2001). Chronic Cu toxicity was also reported in dairy cows (Bradley, 1993). The characteristic feature of industrial Cu intoxication is that the animals are reared close to industrial plants, and ingest Cu from industrial deposits through feed or from the air together with other toxic elements (Bíreš et al., 1991; Elgerwi et al., 1999).

In conclusion it can be said that the soil in the area of Kaľava village is profusely contaminated by heavy metals and Cu was the most abundant metal. The contamination could affect the rumen and hindgut inocula fermentation as well as the protozoan population of grazing sheep. Unfortunately, knowledge in this research area is very limited, and therefore any attempt to rationalize fermentation production losses associated with the non-ferrous metal industry contaminated area is largely speculative.

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