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Original article

In vitro study of the effect of corn dried distillers grains with solubles on rumen fermentation in sheep

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Abstract

The aim of the *in vitro* study was to determine the effect of corn dried distillers grains with solubles (corn DDGS), used as a replacement for the concentrate ingredients of sheep diet, on rumen fermentation. The material for the study was the ruminal fluid of Polish Merino sheep which was incubated during 4-, 8- or 24-hour periods. Five groups of samples were prepared for *in vitro* fermentation: C – control, incubated with the substrate consisting of the concentrate ingredients; D1, D2 and D3, where DDGS was used as a substrate added in proportions of 10, 20 and 30% of dry matter of the concentrate; and D4, where 100% DDGS was used as a substrate. After fermentation, the gas and short chain fatty acids (SCFAs) analyses were performed using gas chromatography. The ammonia concentration and pH were also determined, and the SCFA utilization index (NGR), the fermentation efficiency (FE) and the index of cell yield of ruminal microorganisms (CY) were calculated. This research showed no effect of DDGS on the methane emission. The positive correlations between the amount of methane and ammonia concentrations in the 8- and 24-hour fermentation periods were found. DDGS addition increased propionate proportion, but decreased production of acetate ($p<0.01$). Additionally, D1, D2, D3 and D4 substrates lowered isobutyrate ($p<0.05$) and isovalerate ($p<0.01$) production. Based on the results obtained, it can be stated that partial substitution of the concentrate ingredients with DDGS did not have deleterious effect on sheep rumen fermentation processes.

Key words: sheep, short chain fatty acids, methane emission, ammonia

Introduction

Physiological characteristics of ruminants are anaerobic fermentation processes in the rumen, which lead to the production of inter alia, short chain fatty acids (SCFAs). The SCFA profile, especially the ratio of non-glucogenic SCFAs (acetate, butyrate) to glucogenic SCFAs (propionate), is related to rumen methanogenesis, milk composition and animal energy balance. The total concentration and proportions

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of particular SCFAs in ruminal fluid depend mainly on the composition of diet and conditions inside the rumen (Morvay et al. 2011).

Most greenhouse gases emitted by livestock are the effects of microbial fermentation taking place in the animal rumen and large intestine. Methane is one of them and amounts to about 18% of all the greenhouse gases responsible for global warming (Zhou et al. 2007). Every year animal production releases into the atmosphere 80-115 million tons of methane, which represents 15-20% of anthropogenic emission of methane in the world (Wei-lian et al. 2005). The influence of methane on global warming reinforces tendencies in agriculture to market new feed additives and components of food lowering methanogenesis (McGinn et al. 2009). One of these feed additives is dried distillers grains with solubles (DDGS) considered as the modulator of rumen fermentation profile and inhibitor of methane production. However, most of the research concerning the effect of DDGS on rumen fermentation was performed on cattle (Kleinschmit et al. 2006, Behlke et al. 2008, McGinn et al. 2009, Zhang et al. 2010, Morvay et al. 2011, Morrow 2012, Hünerberg et al. 2013, Segers et al. 2013, Miśta et al. 2014). Research concerning sheep rumen fermentation influenced by DDGS addition is limited (Behlke et al. 2007, 2008, Radev 2012). However, previous studies showed that DDGS affects blood parameters in sheep, milk production in ewes, and rearing parameters in lambs (Dimova et al. 2009, Radunz et al. 2011, Şahin et al. 2013, Westreicher-Kristen et al. 2014).

DDGS is the main by-product of biofuel industry. It was estimated that 1-2 million tons of cereal will be used for bioethanol production in Poland in the next few years. Such throughput will lead to the production of 300-600 thousand tons of DDGS (Zachwieja et al. 2013). Due to its protein and energy supply, using DDGS as the component of fodder has become a way of its natural utilization.

Nowadays, DDGS is one of the most economical and prevalent food components for animals in the United States. High energy value (3674-4336 kcal/kg of dry matter), protein (27-33% of dry matter), lysine (0.6-1.1% of dry matter) and phosphorus (0.57-0.85% of dry matter) contents, as well as high digestibility (50-68%) make DDGS a beneficent component of ruminant and monogastric animal diets (Shurson 2011). Results of other authors; research encouraged us to verify the DDGS effect on the rumen fermentation profile in sheep.

The aim of our *in vitro* research was to examine how corn DDGS used as a replacement for the concentrate ingredients of sheep diet affects rumen fermentation profile.

Materials and Methods

Animals and fermentation substrates

The material for the study was the ruminal fluid of fistulated Polish Merino sheep (n=6), which was withdrawn 1 hour after the morning feeding. Prior to the study, the animals were fed the diet formulated according to the Polish Feeding Standards (1998). The sheep;s commercial concentrate diet mixed with corn DDGS in varied proportions was used as a substrate for *in vitro* fermentation of the ruminal fluid. Five groups of ruminal fluid samples were prepared: C (control), where 1 g of the commercially available concentrate (consisting of corn, extracted soybean meal, barley, wheat bran, dried sugar beet pulp, malt sprouts, calcium carbonate, and sodium chloride) was used as a substrate; D1, D2 and D3, where DDGS was used as a substrate added in proportions of 10, 20 and 30% of dry matter of the concentrate; and D4, where the substrate consisted of 100% DDGS. All substrates were analysed chemically (Table 1).

In vitro fermentation of ruminal fluid

The ruminal fluid samples were mixed with the buffer solution with pH 7.8 (McDougall 1948) in the ratio 1:3 and homogenized. In the obtained suspension pH was measured using CP-401 pH-meter (ELMETRON, Poland) with an EPP-3 electrode and temperature sensor. These samples were centrifuged (15 min, 13 000 rpm) directly after the addition of buffer with the purpose of further analyses. The formic acid was added to these samples (0.1 ml/2 ml of sample) to inhibit the fermentation processes. Additionally, other samples were prepared for *in vitro* fermentation: from each ruminal fluid sample 15 subsamples (5 for each incubation period) were made and assigned to one of the groups examined (C, D1, D2, D3, D4), depending on the substrates added later. Twenty ml of each ruminal fluid sample was put to 125-ml serum bottles (Sigma-Aldrich, USA), four-fold diluted with buffer solution and mixed with 1 g of one of the substrates. Altogether, 90 samples were prepared for incubation, 30 samples with different substrates for each incubation period. The bottles were then thoroughly flushed with carbon dioxide from a pressure bottle and hermetically sealed with a manual crimper. Thus prepared samples were then incubated in a shaking water bath at 39 C for 4, 8 and 24 hours.

Table 1. Chemical composition of the substrates used for *in vitro* fermentation.

Group	Ash %	Crude protein %	Crude fibre %	Crude fat %	NDF %	ADF %	Gross energy MJ/kg
C	6.12	19.31	5.21	1.67	15.56	6.09	15.70
D1	6.04	20.66	5.38	2.93	18.32	6.48	15.16
D2	5.93	20.80	5.59	3.91	19.77	6.35	16.05
D3	5.52	21.89	5.48	4.94	21.1	6.57	16.60
D4	5.01	24.87	8.72	11.2	36.71	11.86	18.05

C – concentrate (control)
D1, D2, D3 – concentrate containing 10%, 20% and 30% corn DDGS in dry matter, respectively, D4 – 100% corn DDGS

Analyses of selected fermentation products

After the incubation, the headspace gas overpressure inside each bottle was measured. The gas samples were analysed for methane content using 7890A gas chromatograph (Agilent Technologies, USA) with TCD and FID detectors.

In the liquid samples pH was measured. These samples were centrifuged (15 min, 13 000 rpm) and the formic acid (0.1 ml/2 ml of sample) was added to them to inhibit the fermentation processes. Both incubated and unincubated samples were analysed using 7890A gas chromatograph with FID detector for the total SCFA and the acetate, propionate, isobutyrate, butyrate, isovalerate, valerate, isocaproate and caproate concentrations.. Identification and the level of SCFAs were assessed by comparison of retention times and area under the peaks with standards (Supelco) using ChemStation software (Agilent Technologies, USA). Based on these results, molar proportions (mol%) of each SCFA in the total SCFA concentration were calculated.

Moreover, in the liquid samples ammonia concentration was determined using modified microdiffusion Conway method with Nessler reagent and Lambda XLS spectrophotometer (Perkin Elmer, USA).

Analysis of substrates

The main nutritional components were estimated in the substrate samples: ash (AOAC Official method 942.05), crude protein (Kjeldahl method, AOAC Official method 984.13, using Kjeltec 2300 Analyzer Unit, Foss), crude fat (AOAC Official method 920.39), crude fibre (AOAC Official method 978.10, using Fibertec 1020, Foss), ADF (AOAC Official method 973.18, using Fibertec 1020, Foss), NDF (JAOAC v. 56, 1352-1356, 1973, using Fibertec 1020, Foss) and gross energy (calorimetrically, using common energy equivalents, FAO, 2003).

Calculations and statistical analysis

The obtained data of SCFAs were used for calculations of the following indices: SCFA utilization (NGR), fermentation efficiency (FE) and the cell yield of ruminal microorganisms (CY).

The SCFA utilization index (NGR), expressed as the ratio of non-glucogenic SCFAs to glucogenic SCFAs, was calculated using the formula of Ørskov (1975), modified by Abrahamse et al. (2008):

$$NGR = (A + 2\,B + Bc) / (P + Bc)$$

where A, P and B represent the molar proportions (mol%) of acetate, propionate and butyrate respectively, and Bc – the valerate and branched-chain fatty acid molar proportion in the total SCFA concentration.

The fermentation efficiency index (FE, expressed as a %) was calculated according to the equation:

$$FE = (0.622\,A + 1.092\,P + 1.56\,B)\,100 / (A + P + 2\,B)$$ (Baran and Žitňan 2002)

where A, P and B represent the molar proportions (mol%) of acetate, propionate and butate, respectively, in the total SCFA concentration.

The index of cell yield of ruminal microorganisms (CY, expressed in g/l) was calculated according to Chalupa (1977):

$$CY = (A + P + B + V)^* 0.03$$

where A, P, B and V represent the concentrations (mmol/l of ruminal fluid) of acetate, propionate, butyrate and valerate, respectively. CY index was calculated on the basis of 30 g of microbial cells/mole of SCFAs.

The results of the study were analysed statistically using two-way ANOVA in the STATISTICA 10 software (StatSoft, USA), according to the following model:

$$Y_{ijk} = \mu + a_i + b_j + (a * b)_{ij} + e_{ijk}$$

where:
Y_{ijk} – the dependent variable under examination
μ – the overall mean
a_i – the effect of the substrate
b_j – the effect of the time
a * b – the fixed effect of the interaction between substrate and time
e_{ijk} – the error term

The differences were analysed at the significance levels of 0.05 and 0.01 and probability values between 0.05 and 0.10 were reported as statistical trends. The Pearson correlation coefficients were calculated for selected parameters.

Results

The data obtained in this *in vitro* study were presented in Table 2. The increasing effect of DDGS used in the substrate on the gas production during *in vitro* ruminal fluid fermentation was shown ($p<0.01$). Total gas together with methane production increased significantly during the incubation time ($p<0.01$). Contrary to our expectations, we did not observe the influence of DDGS on methanogenesis during *in vitro* fermentation of sheep ruminal fluid. However, a small reduction of methane emission (3%) in samples containing 30% of DDGS was found. A positive correlation between DDGS content in the substrate and the total gas production was shown ($p<0.05$; $r = 0.34$). Another positive correlation ($r = 0.42$; $p<0.01$) between the concentration of methane and ammonia emitted in the 8-, but not in the 4-hour fermentation period was noted. Higher correlation coefficient ($r = 0.56$) between ammonia concentration and methane emitted in the 24-hour fermentation period was also stated ($p<0.05$). No effect of DDGS inclusion on ammonia level was observed, but the fermentation time increased ammonia concentration ($p<0.01$). The samples incubated with the substrate containing DDGS had lower pH than the control samples ($p<0.01$). A drop in pH during the incubation time was also observed ($p<0.01$).

The total concentration of SCFAs in fresh ruminal fluid directly after sampling was 92.24 mmol/l (Table 3) and increased during the incubation time ($p<0.01$) (Table 2). The SCFA profile in ruminal fluid of both incubated and unincubated samples is characterised by a high level of acetate, a lower level of propionate and the lowest level of butyrate in the total concentration of these three main SCFAs. The fermentation time and DDGS content in the substrate decreased acetate molar proportion in ruminal fluid ($p<0.01$), which amounted to 78.24 mol% in unincubated samples. The fermentation substrate increased propionate level ($p<0.01$), especially in D3 and D4 samples which contained the highest DDGS content. The butyrate molar proportion was 4.29 mol% in unincubated samples and rose during the incubation time ($p<0.01$). DDGS inclusion in the substrate lowered butyrate level ($p<0.01$). Additionally, the increased DDGS content in the substrate decreased the levels of: isobutyrate ($p<0.05$), isovalerate ($p<0.01$) and caproate ($p<0.01$). The ratio of non-glucogenic to glucogenic SCFAs was 5.69 for unincubated samples and diminished as a result of growing DDGS content in the substrate ($p<0.01$). The substrate had a significant effect on the growth of fermentation efficiency index ($p<0.01$), while the index of cell yield of ruminal microorganisms increased during the fermentation time ($p<0.01$). FE and CY indexes calculated for the unincubated samples amounted to 70.35 % and 2.71 g/l, respectively, and both were lower than those calculated for the incubated samples.

Discussion

Growing DDGS content caused a linear decrease in rumen methanogenesis in both *in vivo* (Benchaar et al. 2013) and *in vitro* studies (Mišta et al. 2014). The research conducted by Behlke (2007) showed that corn DDGS included in diet fed to heifers as a replacement for the forage also lowered the methane emission. However, using corn DDGS as a replacement for corn and corn oil significantly enlarged methane production per milligram of digested substrate during *in vitro* fermentation of heifer ruminal fluid (Behlke 2007). DDGS possesses a greater amount of net energy available for gain relative to corn, which might be caused by a decrease in rumen methane production (Behlke et al. 2008). Surprisingly, the *in vivo* research in lambs showed that DDGS, despite high energy content, resulted in a 29% increase in methane emission (Behlke et al. 2008). Some other authors confirmed the increasing effect of DDGS on the *in vivo* methane production in cattle (Hunerberg et al. 2013) and in poultry (Li et al. 2014). In the present research, we did not observe any significant effects of DDGS addition on methane production during *in vitro* fermentation of sheep ruminal fluid. However, the effect of DDGS on the increase in total gas production was observed in this study. Conversely, earlier *in vitro* research in cows showed a decrease in both methanogenesis and total gas production (Mišta et al. 2014). The greatest drop in gas production was observed in the samples containing 100% DDGS in

Table 2. Effect of time and DDGS inclusion in the substrate on *in vitro* fermentation parameters in sheep rumen.

Fermentation parameters	4 h					8 h					24 h					SEM	*P* value		
	Substrates					Substrates					Substrates								
	C	D1	D2	D3	D4	C	D1	D2	D3	D4	C	D1	D2	D3	D4		substr	time	sb x t
Gas production[1]	42.54	44.06	47.53	87.34	95.50	81.35	77.86	80.31	136.97	130.40	110.21	107.78	99.71	178.09	178.11	6.638	<0.001	<0.001	0.996
Methane[1]	11.91	12.64	13.71	15.38	14.70	23.00	23.02	23.94	23.67	22.85	34.36	33.41	32.35	28.37	37.90	1.402	0.964	<0.001	0.977
Ammonia[1]	30.64	42.80	37.43	21.15	19.50	48.89	24.34	23.49	31.66	36.16	96.60	136.82	168.69	84.31	103.09	8.277	0.702	<0.001	0.789
pH	6.65	6.60	6.59	6.53	6.53	6.45	6.45	6.42	6.30	6.35	6.24	6.26	6.24	5.98	6.11	0.022	<0.001	<0.001	0.449
Total SCFAs[1]	159.0	160.6	121.5	119.3	157.3	201.3	189.2	211.1	216.1	232.3	246.0	228.2	251.2	286.8	255.0	7.058	0.296	<0.001	0.914
SCFAs[2]:																			
Acetate	70.08	68.31	69.05	62.91	61.28	67.85	67.51	68.21	64.55	63.53	63.76	63.69	62.34	61.13	62.16	0.541	0.001	<0.001	0.397
Propionate	19.87	20.37	20.96	26.71	29.66	20.99	21.12	20.79	25.03	27.48	20.84	20.90	21.87	25.57	27.01	0.411	<0.001	0.640	0.645
Isobutyrate	0.73	1.32	0.78	1.20	0.60	0.89	0.81	0.85	0.57	0.60	1.15	1.12	1.14	0.70	0.78	0.047	0.044	0.065	0.173
Butyrate	7.04	7.87	6.65	7.01	6.34	8.01	7.94	7.67	7.42	6.16	10.82	10.61	10.92	9.44	6.95	0.229	<0.001	<0.001	0.080
Isovalerate	1.37	1.37	1.41	1.09	1.16	1.31	1.38	1.28	1.05	1.05	2.09	2.06	2.03	1.47	1.53	0.049	<0.001	<0.001	0.808
Valerate	1.10	1.10	1.11	1.05	0.93	1.17	1.31	1.14	1.33	1.13	1.50	1.42	1.50	1.51	1.46	0.034	0.769	<0.001	0.942
Caproate	0.07	0.08	1.33	0.97	0.03	0.07	0.07	0.06	0.06	0.06	0.20	0.19	0.18	0.17	0.10	0.007	0.006	<0.001	0.195
NGR	3.88	3.76	3.78	2.80	2.45	3.73	3.67	3.86	3.06	2.69	3.77	3.87	3.67	3.01	2.72	0.070	<0.001	0.667	0.881
FE (%)	73.83	74.36	73.77	76.45	77.59	74.41	74.47	73.89	75.71	76.58	75.13	74.65	75.15	76.41	76.65	0.179	<0.001	0.216	0.497
CY[3]	4.64	3.93	3.56	3.00	4.63	5.91	5.48	6.19	6.37	6.85	7.09	6.62	7.28	8.40	7.47	0.189	0.249	<0.001	0.910

C, D1, D2, D3, D4 – substrates compositions were shown in Table 1.
[1] mmol/l of not diluted ruminal fluid
[2] mol/100 mol of total SCFA concentration (mol%)
[3] g/l of not diluted ruminal fluid

Table 3. Fermentation profile in fresh ruminal fluid of sheep (unincubated samples).

	pH	SCFA[1]	Acetate[2]	Propionate[2]	Isobutyrate[2]	Butyrate[2]	Isovalerate[2]	Valerate[2]	NGR	FE (%)	CY[3]	Ammonia[1]
$\bar{x}$	7.92	92.24	78.24	14.73	0.84	4.29	1.28	0.63	5.69	70.35	2.71	24.45
SEM	0.044	6.040	0.393	0.447	0.071	0.183	0.066	0.039	0.190	0.197	0.179	4.627

[1] mmol/l of not diluted ruminal fluid
[2] mol/100 mol of total SCFA concentration (mol%)
[3] g/l of not diluted ruminal fluid

the substrate compared to control samples. Other authors' research did not confirm the effect of DDGS on the total gas production during rumen fermentation in both *in vitro* (Segers et al. 2013) and *in vivo* conditions (Morrow 2012).

Ammonia is another important end-product of rumen fermentation emitted by animals. In the present research, no significant effect of DDGS on the ammonia production during *in vitro* rumen fermentation was observed. Only the sample containing 30% of DDGS showed a slight decrease in ammonia level as compared to the control samples. Other authors did not show any significant DDGS effects on ammonia concentration in rumen of young steers (Leupp et al. 2009). However, Radev (2012) found that dietary supplementation with DDGS increased rumen ammonia concentration in sheep. Similar effect was reported by Loy et al. (2007) in the *in vivo* study in heifers. Converse results were obtained by Li et al. (2014) who proved that feeding DDGS to laying hens resulted in 14% fall in ammonia emission. The above mentioned examples show diverse impact of DDGS on the ammonia emission in different species.

A stable physiological condition of the rumen is necessary to maintain high fermentation efficiency. The pH optimal for microorganisms existing in the rumen ranges between 6.2 and 6.6 (Veth and Kolver 2001). In the present study, the effect of DDGS on ruminal pH was observed; however, all results except D3 sample in the 24-hour fermentation period were in the above mentioned range. Stable pH value is beneficial for fermentation processes since ruminal microorganisms need relatively constant pH in order to function properly. The pH dropping slightly below 6.0 caused a decrease in the methanogenesis and ammonia production (Lana et al. 1998). In our study, a slight decrease in methanogenesis was found in group D3 in the 24-hour fermentation period, where the lowest pH was observed. The effect of the fermentation time on pH value in the analysed samples was noticed, and it corresponds to earlier research (Miśta et al. 2014).

Short chain fatty acids, produced in the rumen by microorganisms carrying out fermentation processes, are used by ruminants as the main energy source (Morvay et al. 2011). The previous study in cows showed a decrease in total SCFA concentration in ruminal fluid under the influence of DDGS content in the fermentation substrate (Miśta et al. 2014). In lambs, the kcal energy available from the SCFAs produced per milligram of digested DM decreased as corn was replaced with DDGS (Behlke et al. 2008). In the present study, DDGS inclusion did not affect the total SCFA level.

The *in vitro* research in cattle conducted by Kleinschmit et al. (2006) showed that mutual molar proportions of acetate to propionate did not change due to corn DDGS addition. Similarly, replacement of silage or barley grain with DDGS did not affect the total production and changes in the profile of SCFAs in the rumen of cows (Zhang et al. 2010). The results of other studies showed that increasing DDGS content in the fermentation substrate lowered acetate with an increase in propionate and butyrate rumen production (Behlke et al. 2007, Loy et al. 2007). The *in vitro* studies showed that the use of DDGS decreased acetate and propionate levels in lambs (Behlke 2007). Similarly, DDGS decreased acetate and increased propionate molar proportions in the total SCFA concentration in the present study. The *in vitro* research in cows showed the influence of DDGS inclusion on a decrease in total SCFA level as well as isobutyrate and isovalerate molar proportions, but no changes in acetate, propionate and butyrate proportions were observed (Miśta et al. 2014). Similarly, Zhang et al. (2010) also observed iso-acids decrease under DDGS influence. The results obtained by the aforementioned authors correspond with the present results, where a decrease in isobutyrate and isovalerate production in the samples incubated with DDGS was also noted.

The non-glucogenic to glucogenic SCFA ratio affects, inter alia, methanogenesis, milk composition and energy balance in animals (Morvay et al. 2011). *In vitro* research showed that 20-80% inclusion of corn to the substrate dry matter decreased NGR index (Rymer and Givens 2002). In the present study, corn-derived DDGS also caused NGR decrease: all values are in the ranges of results obtained by other authors (2.68-4.45). The previous research in cows did not demonstrate significant effects of DDGS on FE and CY indexes (Miśta et al. 2014). In the present study, DDGS addition caused an increase in FE index. Higher DDGS inclusion (D3 and D4) also caused CY increase in the 8- and 24-hour fermentation periods. These changes are beneficial and indicate growth in SCFA production under the influence of DDGS.

Differences between literature data can be caused by different DDGS composition, which may result from variability in drying processes and storage. For example, using too high temperatures to heat DDGS caused a formation of Maillard reaction products with low bioavailability, and sometimes also toxic compounds (De Almeida 2013). The substitution of different foods (concentrate or forage components) with DDGS may also cause different effects.

Summing up, we can conclude that the growth in propionate production, together with the fall in acetate and butyrate level indicate the positive effect of DDGS on the *in vitro* fermentation profile in the pres-

ent study. Lack of changes in methanogenesis and ammonia level during *in vitro* fermentation confirmed that DDGS did not have deleterious effect on rumen fermentation processes in sheep. However, final evaluation of the effect of DDGS on the microbial fermentation in sheep rumen should be verified by *in vivo* studies.

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