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In Vitro Ruminal Fluid Fermentation as Influenced by Corn-Derived Dried Distillers' Grains with Solubles*

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This study was conducted to evaluate changes to *in vitro* ruminal fluid fermentation due to the use of corn-derived dried distillers' grains with solubles (corn DDGS) as a partial or complete replacement for crushed cereal and oilseed meals in the fermentation substrate. The control substrate consisted of mixed cereal and oilseed meals (barley, wheat, soybean and rapeseed), while the experimental substrates were the same meals with increasing portions replaced with corn DDGS. Including corn DDGS decreased the total VFA concentration ($P<0.05$), ammonia level ($P<0.001$), methane emission ($P<0.05$) and total gas production ($P<0.001$) during microbial fermentation. Using DDGS-containing substrates did not change the proportions of acetate, propionate and butyrate, but did decrease the proportions of isobutyrate and isovalerate ($P<0.001$). The fermentation efficiency, VFA utilization index, cell yield coefficient and pH of the ruminal fluid also remained unchanged. The partial replacement of cereal and oilseed meals with corn DDGS had no deleterious effects on ruminal fluid fermentation.

Key words: Microbial fermentation; rumen; distillers' grains; methane; volatile fatty acids; ammonia.

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Dried distillers' grains with solubles (DDGS) are the main co-product of ethanol production. They have become much more available in recent years due to the expansion of fuel ethanol production. DDGS are a good source of energy. They also have a high protein content (28-36% of the DM), of which a considerable portion is ruminally undegradable protein. Therefore, they are used in animal feed, particularly for dairy cattle (SCHINGOETHE *et al.* 2009). The wet form can also be used but is difficult to store and must be used within a few days of production.

The starch in corn grain is fermented during ethanol production. Most of the other grain constituents, such as the original fatty acids, protein and phosphorus, remain unchanged. Thus, corn-derived dried distillers' grains with solubles (corn DDGS) contain little starch and more protein, fat and biologically available phosphorus than corn (STALLINGS 2009). The starch content in the DM of corn DDGS ranges from 4.7 to 5.9%, compared to 70.6 to 71.8% in corn samples (BELYEA *et al.* 2004). DDGS are also a source of highly degradable fiber (AL-SUWAIEGH *et al.* 2002). Their high

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NDF content, ranging from 32 to 44% (ANDERSON *et al.* 2006; KLEINSCHMIt *et al*. 2006) means that DDGS may be used as a partial replacement for forage and feed concentrates in the diets of dairy cattle (ZHANG *et al.* 2010a,b). However, for lactating cows, it is recommended that DDGS are only used to partially replace the concentrate, and not as a replacement of forage ingredients in the diet, because adequate effective fiber is needed to avoid milk fat depression (SCHINGOETHE *et al.* 2009). Moreover, DDGS are rich in B vitamins because they contain dried yeast cells from the distillery process.

Corn DDGS has a lower energy value than grain because the main energy source in corn grain is starch which is mostly fermented to ethanol in the biofuel production process. Fat and NDF remain as sources of energy in corn DDGS (ZACHWIEJA *et al.* 2013). Wheat DDGS provide at least equal energy to barley DDGS when used as a replacement for moderate amounts of grain in finishing diets for cattle (GIBB *et al*. 2008).

Currently, DDGS are under consideration as a partial concentrate substitution in dairy cow diets in their dry period in order to lower the dietary cation-anion difference. This should diminish post-parturient hypocalcemia. We hypothesized that DDGS would have no deleterious effects on the microbial fermentation of ruminal fluid from dry cows and that some parameters might even be improved. The aim of this *in vitro* study was to determine the effects on ruminal fluid fermentation of partially replacing the crushed cereal and oilseed meals in the substrate with corn DDGS. In particular, the influence on methane production, pH, volatile fatty acid (VFA) levels, and ammonia concentration was assessed.

Material and Methods

Experimental animals

The study used eight lactating Polish Holstein dairy cows in their dry period (before their first, second or third calving). The animals were housed in an open housing system and had been fed a basal diet (total mixed ration – TMR) prior to the study (g/kg of DM): 521.8 maize silage, 91 wilted grass silage, 20 straw, 72.5 beet pulp silage, 94 cereal meal (50% wheat, 50% barley), 95.1 extracted rapeseed meal, 76.1 extracted soybean meal, 9.7 mineral mixture, 9.7 sodium bicarbonate, 5.8 chalk, 4.3 protected fat, 0.1 dried yeasts. They were selected from the herd using the analogue method based on their age and milk yield during the previous lactation.

Their diet was formulated according to the French standard INRA (IZ-INRA 2009). Cows were allowed free access to water. All of the procedures were performed with the approval of the Second Local Ethics Commission for Experiments on Animals in Wroclaw, Poland (license no. 166/2010).

Substrate composition

The *in vitro* study was performed as part of an *in vivo* experiment where cows received balanced feed with a corn DDGS content of 0, 10, 15 or 20% of the dry matter. The substrates for the *in vitro* study were formulated on the basis of the concentrate ingredients. The control substrate (C) contained the same ingredients as the concentrate in the basal diet (barley and wheat meal, extracted rapeseed meal, extracted soybean meal). The D1, D2 and D3 substrates contained the same ingredients but with DDGS added in proportions calculated to reflect those in the *in vivo* diets. The D4 substrate was entirely composed of DDGS. The ingredients, chemical compositions and nutritional values of the *in vitro* substrates are presented in Table 1.

The substrates were analyzed using standard methodologies (AOAC 1997 and 2005) for dry matter (AOAC 2005, Official method 934.01), crude protein (KJELDAHL method, AOAC 2005, Official method 984.13), ether extract (AOAC 2005, Official method 920.39), ash (AOAC 2005, Official method 942.05), NDF (neutral detergent fiber) assayed without a heat stable amylase and expressed inclusive of residual ash (AOAC 1997, Official method 984.13) and ADF (acid detergent fiber) expressed inclusive of residual ash (AOAC 1997, Official method 984.13). NEL (net energy of lactation, MJ) was determined according to the INRA feeding system (IZ-INRA 2009). The content of NSC (nonstructural carbohydrates) was calculated according to NATIONAL RESEARCH COUNCIL (2001): 1000-(Ash + CP + EE + NDF), where ash, crude protein (CP), ether extract (EE) and neutral detergent fiber (NDF) contents were expressed as g/kg of dry matter (DM).

In vitro fermentation

Using a probe, ruminal fluid was collected from cows 3 weeks before calving, 2 hours after their morning TMR feed. The samples were filtered through 2 layers of cheesecloth and their pH was measured using an CP-401 pH-meter (Elmetron, Poland) with an EPP-3 electrode and temperature sensor. Fifteen subsamples of 30 ml each were taken from each animal's sample. Each subsample was diluted threefold with a buffer solution (MCDOUGALL 1948) and transferred into a 125-ml serum bottle. A 1 g sample of the C, D1, D2, D3 or D4 substrate was added to each bottle, in triplicate for the ruminal fluid collected from each cow. The

Table 1

Ingredients, chemical compositions and nutritional values of substrates for the *in vitro* experiment

Item	C	D1	D2	D3	D4
Cereal meal (50% barley, 50% wheat) (g/kg)	357	168	74	0	0
Extracted rapeseed meal (g/kg)	357	328	191	141	0
Extracted soybean meal (g/kg)	286	146	147	85	0
Corn DDGS (g/kg)	0	358	588	774	1000
Dry matter (g/kg)	880.0	894.2	903.2	910.4	918.2
Organic matter (g/kg)	838.3	847.2	853.7	859.1	868.1
Crude protein (g/kg of DM)	355.6	338.3	329.6	315.2	270.9
Ether extract (g/kg of DM)	12.8	52.5	78.2	98	122
NDF (g/kg of DM)	266.7	323.9	348.0	373.8	399.8
ADF (g/kg of DM)	120.2	137.5	133.7	137.4	129.2
Ash (g/kg of DM)	47.4	52.6	54.8	56.4	54.6
NEL[a] (MJ/kg of DM)	7.47	7.33	7.33	7.26	7.26
NSC[b] (g/kg of DM)	317.5	232.7	189.4	156.6	152.7

[a]net energy of lactation
[b]nonstructural carbohydrates: 1000-(ash+ether extract+crude protein+NDF)

bottles were thoroughly flushed with carbon dioxide to obtain anaerobic conditions, and hermetically sealed with a manual crimper. The incubation was performed in a shaking water bath at 39ºC for 4, 8 and 24 hours. At the end of incubation, the fermentation gas was sampled with a gas-tight syringe for analyses. Additionally, before the gas analysis, the headspace pressure inside each bottle was measured. In the remaining liquid sample, fermentation was stopped by adding 0.05 ml formic acid/ml of sample.

Chemical analyses

Gas samples were analyzed for methane using an 7890A gas chromatograph (Agilent Technologies, USA) with a flame ionization detector and thermal conductivity detector, two Supelco columns: Porapak Q (length: 1.83 m, ID: 2.1 mm) and HayeSep Q (length: 1.83 m, I.D.: 2.1 mm), as well as 5A Molecular Sieve. Helium was used as the carrier gas (flow: 25 ml/min). A standard curve for methane was prepared using gas mixtures certified for analyses (Linde; Agilent Technology). Based on the gas pressure and the methane percentage of the total gas volume, the molar concentration of methane was calculated using the Clapeyron equation.

Liquid samples were centrifuged (2800 g for 20 min) and analyzed on the 7890A gas chromatograph (Agilent Technologies, USA) with a flame ionization detector and Agilent J&W column DB-23 (length: 60 m, ID: 0.25 mm), with helium as the carrier gas (flow: 25 ml/min). This analysis was done to determine the total VFA and acetic, propionic, isobutyric, butyric, isovaleric, valeric, isocaproic and caproic acid concentrations. Standard curves were prepared using the Supelco Volatile Free Acid Mix and the concentration of each acid was calculated. The ammonia in the samples was separated by microdiffusion in Conway units and determined with a Nessler reagent using a Lambda XLS spectrophotometer (Perkin Elmer, USA) at a wavelength of 410 nm. The pH value was measured in all of the samples.

Calculations and statistical analysis

Based on the results of the VFA analysis, the fermentation efficiency (FE) was calculated according to the equation of ØRSKOV (1975) as modified by BARAN & ITŇAN (2002):

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

where A, P and B, respectively, represent the molar proportions (mol%) of acetic, propionic and butyric acids in the total VFA concentration. Expressed as a %.

The VFA utilization index (NGR) was expressed using the non-glucogenic VFA to glucogenic VFA ratio, according to ØRSKOV (1975), as modified by ABRAHAMSE *et al.* (2008):

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

where A, P and B, respectively, represent the molar proportions (mol%) of acetic, propionic and butyric acids and Bc is the valerate and branched-chain fatty acid molar proportion in the total VFA concentration.

The index of cell yield of mixed ruminal organisms was calculated according to CHALUPA (1977):

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

where: A, P, B and V, respectively, represent the concentrations (mmol/l of ruminal fluid) of acetic, propionic, butyric and valeric acids. The calculations are based on 30 g of microbial cells/mole of VFAs. Expressed in g/l.

Data were subjected to a two-factorial ANOVA using the STATISTICA 10 software package according to the following model:

$$Y_{ijk} = \mu + s_i + t_j + (s * t)_{ij} + e_{ijk}$$

where Y_{ijk} is the dependent variable under examination, μ is the overall mean, s_i is the substrate effect, t_j is the effect of fermentation time, s * t is the fixed effect of the interaction between substrate and fermentation time, and e_{ijk} is the error term. Significance was declared at $P<0.05$.

Results

The effects of adding corn DDGS to the substrate on *in vitro* fermentation parameters are presented in Table 2. The volumes of gas produced during the fermentation processes significantly decreased with increasing proportions of added DDGS ($P<0.001$). Similarly, increasing the DDGS supplementation decreased methane emissions per liter of ruminal fluid ($P<0.05$). The inclusion of DDGS in the concentrate caused about a 28% reduction in methanogenesis during *in vitro* fermentation.

A depreciative effect of DDGS on the total VFA concentration was observed ($P<0.05$). No significant changes were seen in the proportions of the three most important acids (acetic, propionic and butyric). However, a slight declining propensity for acetate and increase for propionate were noted as the DDGS inclusion level increased. The other indices calculated on the basis of VFAs (NGR, FE and CY) also showed no significant change due to the presence of corn DDGS, but higher levels of DDGS led to slightly greater fermentation efficiency and a lower non-glucogenic to glucogenic VFA ratio. The index of cell yield tended to be smaller with higher DDGS levels. The percentages of isobutyrate and isovalerate diminished after DDGS addition ($P<0.01$ and $P<0.05$, respectively).

Table 2

Effect of different DDGS inclusion levels on ruminal parameters after 4, 8 and 24 hour *in vitro* fermentation

	4 h					8 h					24 h					SEM	Significance		
	DDGS level					DDGS level					DDGS level						time	substrate	interaction
	C	D1	D2	D3	D4	C	D1	D2	D3	D4	C	D1	D2	D3	D4				
Gas production[b]	48.31	42.95	36.43	32.45	25.21	60.32	50.07	41.62	35.59	29.55	88.08	76.98	63.94	61.17	53.44	2.051	***	***	NS
Methane[b]	7.27	6.48	5.61	4.98	4.57	10.02	9.88	8.81	7.93	7.17	17.64	16.94	13.71	14.03	12.10	0.510	***	*	NS
pH	6.64	6.69	6.71	6.72	6.74	6.56	6.61	6.63	6.64	6.65	6.50	6.54	6.53	6.53	6.51	0.016	***	NS	NS
Total VFA[b]	164.7	164.5	144.5	141.7	142.3	192.3	210.2	175.3	185.0	173.4	274.9	262.0	224.4	223.5	202.6	5.92	***	*	NS
VFA[c]:																			
Acetate	54.59	52.49	51.50	49.85	49.35	51.33	51.47	50.37	48.63	47.79	50.28	51.38	49.50	50.80	51.17	0.548	NS	NS	NS
Propionate	31.77	32.99	33.38	35.27	34.82	33.58	32.85	33.58	34.08	35.27	31.51	31.04	32.86	32.50	33.36	0.378	NS	NS	NS
Isobutyrate	0.55	0.58	0.54	0.49	0.47	0.66	0.66	0.58	0.59	0.55	1.00	0.88	0.87	0.79	0.65	0.021	***	**	NS
Butyrate	9.19	9.66	10.04	9.79	10.55	9.50	9.95	10.40	10.96	10.70	10.68	10.59	10.26	9.92	9.32	0.159	NS	NS	NS
Isovalerate	1.02	0.94	0.95	0.83	0.86	1.37	1.36	1.08	1.09	0.99	2.29	2.00	1.90	1.73	1.49	0.059	***	*	NS
Valerate	2.51	2.98	3.20	3.33	3.47	3.18	3.25	3.55	4.12	4.17	3.73	3.64	4.05	3.70	3.46	0.157	NS	NS	NS
Isocaproate	0.02	0.01	0.01	0.01	0.01	0.02	0.02	0.02	0.02	0.02	0.01	0.01	0.01	0.04	0.02	0.002	NS	NS	NS
Caproate	0.35	0.35	0.39	0.43	0.46	0.37	0.45	0.43	0.51	0.51	0.49	0.46	0.54	0.51	0.57	0.019	*	NS	NS
NGR	2.21	2.12	2.05	1.91	1.97	1.99	2.07	2.03	1.99	1.87	2.11	2.13	1.96	2.02	1.93	0.038	NS	NS	NS
FE (%)	79.18	79.93	80.20	81.01	80.93	80.29	80.04	80.42	80.85	81.34	79.85	79.52	80.37	80.06	80.61	0.192	NS	NS	NS
CY[d]	4.82	4.84	4.25	4.34	4.19	5.63	6.14	5.14	5.07	5.10	7.93	7.60	6.51	6.66	6.63	0.177	***	NS	NS
Ammonia[b]	5.51	3.89	3.34	3.83	2.87	14.55	10.44	9.01	6.81	4.39	124.64	113.00	47.04	33.78	22.30	4.519	***	***	***

C, D1, D2, D3, D4 – See Table 1 for substrate compositions; SEM – standard error of the mean; [a]Effects of fermentation time, substrate and the interaction between substrate and fermentation time: NS – not significant, * $P<0.05$, ** $P<0.01$, *** $P<0.001$; [b]mmol/l of undiluted ruminal fluid; [c]mol/100 mol of total VFA concentration; [d]g/l of undiluted ruminal fluid.

The proportions of these iso-acids decreased ($P<0.001$) and the proportion of caproate increased ($P<0.05$) over the course of incubation.

A remarkable decrease in ammonia production was also observed with increasing corn DDGS contents ($P<0.001$). The ammonia concentration diminished by 28% for D1 after 4 hours of fermentation up to even 82% for D4 after 24 hours. DDGS contents did not affect the pH of the incubated samples was and its mean value was 6.6.

All the parameters expressed as concentration per liter of ruminal fluid, such as gas production, methane, total VFA concentration and ammonia, increased during the 24-hour incubation period. These products of microbial activity are not absorbed in the model of *in vitro* fermentation. The microbial cell yield calculated on the basis of VFA concentration increased during the experimental period ($P<0.001$). Except for ammonia ($P<0.001$), no substrate/time interactions were detected for the analyzed parameters.

Discussion

Volatile fatty acids are produced in the rumen via microbial fermentation and they serve as the main energy source for ruminants (MORVAY *et al.* 2011). Their proportions in the ruminal fluid depend on the composition of the diet and the conditions in the rumen.

Similarly to the results of our *in vitro* research, KLEINSCHMIT *et al.* (2006) reported a lower total concentration of VFAs in cows fed DDGS from different sources (at 20% of diet DM, when DDGS replaced a portion of soybean meal) compared to the control diet. Greater VFA production in cows fed the control diet might be due to the presence of more nonstructural carbohydrates, which allow more fermentation to occur. In our experiment, the reduction in dietary nonstructural carbohydrates due to the partial replacement of crushed cereal meal, soybean meal and rapeseed meal with corn DDGS may have meant less VFA production. According to HAM *et al.* (1994), replacing starch (grain) with a combination of highly digestible fiber, fat and protein (corn distillery byproducts) should reduce the total amount of acids produced in the rumen, thereby reducing the potential for subacute acidosis.

Neither acetate nor propionate molar proportions were significantly affected by adding DDGS in the experiment conducted by KLEINSCHMIT *et al.* (2006). These results are similar to ours, but higher butyrate proportions were observed by these authors in the case of DDGS diets. As in our study, after the addition of DDGS, a decrease in isobutyrate and a tendency towards a decrease in isovalerate levels were also noted. Similar results for the isobutyrate and isovalerate molar proportions were obtained by ZHANG *et al*. (2010a) as a result of the partial substitution of wheat DDGS for barley silage in the diet of dairy cows.

The non-glucogenic to glucogenic VFA ratio is associated with effects on methane production, milk composition, and energy balance (MORVAY *et al*. 2011). Glucogenic propionate contributes to energy deposition in body tissues, whereas non-glucogenic acetate and butyrate are sources for long-chain fatty acid synthesis. Higher NGR was related to a higher milk fat content (ABRAHAMSE 2009). Too high NGR indicates a high loss of energy in the form of gases (ØRSKOV 1975). In our study, despite the different carbohydrate sources, including DDGS did not change this coefficient compared to the value for the control substrate.

Fermentation efficiency based on VFA production is very useful for analyzing the effect of some feed additives on ruminal fluid fermentation via microbial metabolism modulation. Since the energy in acetate, propionate and butyrate is respectively 62, 109 and 78% of that in fermented hexose, metabolically useful energy recovered in fermentation end-products can be increased by enhancing the production of propionate and to a lesser extent butyrate at the expense of acetate production (BARAN & ITŇAN 2002). In our study, including corn DDGS did not increase FE significantly, but the decrease in methane production suggests that the loss of energy was lower.

Inhibiting methanogenesis is very desirable. Ruminal production of methane causes a 3 to 12% loss of feed gross energy. Furthermore, its emission contributes to the greenhouse effect. Replacing forage (brome hay) with DDGS reduced *in vitro* methane production in the ruminal fluid of heifers without affecting the amount of energy produced in the form of VFAs (BEHLKE *et al.* 2007). This effect could be caused by the higher fiber content in brome hay compared to DDGS. In our research, when DDGS partially or fully replaced the concentrate, the production of methane also decreased, although the fermentation efficiency calculated on the basis of energy produced in the form of VFAs remained unchanged. The higher fat content in DDGS compared to mixed meals could be the reason for the methanogenesis reduction in our experiment, since certain fatty acids reduce ruminal methane release (ZAWADZKI 1993; DOHME *et al.* 2001).

In our study, lower methane emission was accompanied by a decrease in the total ruminal gas production. Together with the reduction in VFA production, this may indicate a decrease in nutrient fermentability due to their being fewer nonstruc-

tural carbohydrates in DDGS than in the applied mixed meals. LIA *et al.* (2012) observed that methanogenic Archaea populations were not affected by the addition of wheat DDGS. Moreover, BEHLKE *et al.* (2008) reported no effect on the total amount of produced methane when DDGS replaced corn as a substrate for *in vitro* ruminal fluid fermentation in heifers.

Only about half of the dietary protein of ruminants is digested in the rumen. Some are readily digestible by intestinal digestion systems, e.g. the proteins in corn, or those treated by heating or the addition of formaldehyde so that they pass through to the abomasum. The manufacturing process of DDGS may damage a portion of the protein due to excessive heat during drying, thus making it unavailable to the animal (KLEINSCHMIT *et al*. 2006).

Since DDGS is a rich source of rumen undegradable protein, ruminal proteolytic activity is expected to be lower for DDGS than for cereal meals, which could result in a decrease in ammonia production, as observed in our experiment. Similarly, KLEINSCHMIT *et al.* (2006) found a lower concentration of ruminal ammonia in cows fed DDGS as a partial substitution for soybean meal than in cows fed the control diet, which coincided with a decrease in the concentration of milk urea nitrogen.

Microbial crude protein is one of the major contributors to the supply of amino acids that are used for milk protein synthesis. Therefore, it is important to accurately evaluate the effect of diet on microbial growth in the rumen (CASTILLO LOPEZ 2012). The low concentrations of ruminal ammonia combined with lower concentrations of nonstructural carbohydrates from DDGS may limit ruminal microbial synthesis, thus further explaining the decline in milk protein content in animals fed DDGS (KLEINSCHMIT *et al.* 2006).

In our study, the cell yield calculated on the basis of VFA concentrations was not significantly affected by the inclusion of corn DDGS, but there was a slight course downward. Different results were reported by LIA *et al.* (2012), who observed greater production of bacterial protein when wheat DDGS replaced a portion of barley grain in the substrate for *in vitro* ruminal fluid fermentation.

Despite the lower VFA production when DDGS were added to the substrate, no significant increase in the pH of rumen samples was observed in our study. A similar lack of change in the ruminal pH after the replacement of grain and silage with wheat DDGS was reported by LI *et al.* (2011). Similarly, ZHANG *et al.* (2010b) reported no increase in rumen pH when barley grain was replaced with DDGS in dairy cow diet, although it tended to be slightly higher after DDGS supplementation, as in our results.

Conclusions

Partial replacement of crushed cereal, soybean and rapeseed meals in the fermentation substrate with corn DDGS decreases the total VFA concentration, methane emission and total gas production. This may be due to the presence of more nonstructural carbohydrates in the concentrate components, which allows more fermentation to occur. Substituting a cereal substrate composed predominantly of starch with a more fibrous substrate such as DDGS resulted in less rapid microbial fermentation taking place in ruminal fluid, which may help mitigate ruminal acidosis. The low concentration of ruminal ammonia could be due to lower ruminal proteolytic activity thanks to the high ruminally undegradable protein content in DDGS, which makes the protein more available for intestinal digestion systems. The replacement of crushed cereal, soybean and rapeseed meals with corn DDGS had no deleterious effects on ruminal fluid fermentation.

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