



Fortification of dried distillers grains plus solubles with grape seed meal in the diet modulates methane mitigation and rumen microbiota in Rusitec

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ABSTRACT

The role of dried distillers grains plus solubles (DDGS) and associative effects of different levels of grape seed meal (GSM) fortified in DDGS, used as both protein and energy sources in the diet, on ruminal fermentation and microbiota were investigated using rumen-simulation technique. All diets consisted of hay and concentrate mixture with a ratio of 48:52 [dry matter (DM) basis], but were different in the concentrate composition. The control diet contained soybean meal (13.5% of diet DM) and barley grain (37%), whereas DDGS treatments, unfortified DDGS (19.5% of diet DM), or DDGS fortified with GSM, either at 1, 5, 10, or 20% were used entirely in place of soybean meal and part of barley grain at a 19.5 to 25% inclusion level. All diets had similar DM, organic matter, and crude protein contents, but consisted of increasing neutral detergent fiber and decreasing nonfiber carbohydrates levels with DDGS-GSM inclusion. Compared with the soy-based control diet, the unfortified DDGS treatment elevated ammonia concentration (19.1%) of rumen fluid associated with greater crude protein degradation (~19.5%). Methane formation decreased with increasing GSM fortification levels ($\geq 5\%$) in DDGS by which the methane concentration significantly decreased by 18.9 to 23.4 and 12.8 to 17.6% compared with control and unfortified DDGS, respectively. Compared with control, unfortified DDGS decreased butyrate proportion, and GSM fortification in the diet further decreased this variable. The proportions of genus *Prevotella* and *Clostridium* cluster XIVa were enhanced by the presence of DDGS without any associative effect of GSM fortification. The abundance of methanogenic archaea was similar, but their composition differed among treatments; whereas

Methanospaera spp. remained unchanged, proportion of *Methanobrevibacter* spp. decreased in DDGS-based diets, being the lowest with 20% GSM inclusion. The abundance of *Ruminococcus flavefaciens*, anaerobic fungi, and protozoa were decreased by the GSM inclusion. As revealed by principal component analysis, these variables were the microorganisms associated with the methane formation. Grape seed meal fortification level in the diet decreased DM and organic matter degradation, but this effect was more related to a depression of nonfiber carbohydrates degradation. It can be concluded that DDGS fortified with GSM can favorably modulate ruminal fermentation.

Key words: dried distillers grains plus solubles, grape seed meal fortification, ruminal fermentation, ruminal microbiota, methane mitigation

INTRODUCTION

Incorporation of industrial by-products in animal diets is an economically and environmentally viable practice for livestock production, especially for ruminants that can take advantages of fiber-rich and low-quality feedstuffs. Dried distillers grains plus solubles (DDGS) are a by-product of ethanol production by yeast fermentation of grain starch. The fermentation process removes starch of grains and, in turn, enriches the content of other nutrients, making DDGS an excellent source of protein, energy, and nonforage fiber in cattle diets (Abdelqader et al., 2009). However, DDGS can alter the characteristics of the diet because DDGS is low in physically effective fiber (small particle size of high specific density) and the NDF of DDGS is highly digestible (Zhang et al., 2010a). Such dietary characteristics may negatively alter ruminal fermentation and rumen health (Li et al., 2012b; Zebeli et al., 2012). Supporting this notion, some studies report that DDGS resulted in undesired changes in ruminal fermentation characteristics (Loy et al., 2007; Li et al., 2012b). In dairy cows, DDGS replacing barley silage decreased

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ruminal pH, rumination, chewing activity, and milk fat percentage (Penner et al., 2009; Zhang et al., 2010a,b). Environmentally, DDGS can lead to increased ruminal ammonia concentration and total N excretion in cattle (Hünerberg et al., 2013).

Due to the antimicrobial properties of plant secondary compounds, previous research attempted to use plant secondary compounds to lower ruminal protein degradation as well as to decrease greenhouse gas emissions in cattle fed DDGS; some success in those studies has been reported (Hao et al., 2011; Li et al., 2012b). In ruminant nutrition, tannins are of interest when supplied in small amounts. For example, in addition to their effects on microbes and mitigating methane emissions (Jayanegara et al., 2010), tannins are also able to build indigestible complexes with certain protein and carbohydrate fractions under the rumen condition, thus decreasing the rate of ruminal degradation and subsequently increasing the flow of these nutrients for the lower gut digestion (Patra and Saxena, 2011). Grape seed meal (**GSM**), the residue from grape seeds after oil extraction, is a rich source of tannin phenols (Shi et al., 2003), making GSM an interesting by-product to be used in diets containing high energy and protein ingredients such as DDGS. We hypothesized that incorporating GSM in the DDGS component of the diet may favorably modulate ruminal fermentation in a way that GSM phenols affect the activity of ruminal microbiota by interacting with various protein and carbohydrate fractions of the DDGS or other components of the diet. Hence, this could shift substrate availability and potentially shape substrate preferences of rumen microbiota. In the present study, we evaluated effects of DDGS with or without graded levels of GSM, used entirely in place of soybean meal and part of barley grain, on the abundance of rumen microbiota and ruminal fermentation characteristics *in vitro* using the rumen-simulation technique (**Rusitec**).

MATERIALS AND METHODS

Treatments and Experimental Diets

Six dietary treatments were used; all containing 48% second-cut meadow hay and 52% concentrate mixture (DM basis; Table 1). The concentrate mixture differed in its composition among diets. The control diet contained 37% barley grain, 13.5% soybean meal, and 1.5% mineral-vitamin mix in diet DM. For the second diet, the entire portion of soybean meal and part of barley were replaced with DDGS (ActiProt, Agrana Stärke GmbH, Tulln, Austria) at a level of 19.5% in total diet DM. For the other 4 diets the same substitution manner was performed but DDGS fortified with GSM, either at

1, 5, 10, or 20% GSM, were used instead of pure DDGS, respectively. The GSM-fortified DDGS products were provided by E. Taufrazthofer (Vinolis Traubenkernöl, Gumpoldskirchen, Austria). To keep all 6 diets at similar OM (~92%) and CP (~16.6%) levels, amount of the 4 DDGS-fortified products was 19.5, 20.5, 22.0, and 25% in diet DM, respectively. Before use, hay was chopped to about 1-cm in length, whereas the concentrate ingredients were ground through a 4-mm sieve. Grape seed meals used were a mixture of red and white grapes (40 and 60% wt/wt, respectively). As analyzed, both GSM contained total phenols 38 to 39 mg/g of DM, which was in a similar range as a previous report (Shi et al., 2003). More details regarding chemical composition of the diet ingredients are illustrated in Table 1. The analyzed chemical composition of the individual ingredients was used to formulate the diets.

Experimental Design, Rusitec Procedure, and Sample Collection

Two Rusitec systems, each consisting of 6 incubation units, thus the experimental units, with an effective volume of 800 mL, were used in this experiment. The experiment was a completely randomized design, whereby 6 diets were tested in 3 experimental runs with 2 replicates in each run, resulting in 6 independent measurements per each treatment. The procedure of Rusitec is explained in details in a previous study (Klevenhusen et al., 2014). In brief, each experimental run lasted 10 d, whereby the first 5 d were used for equilibration of the system and the last 5 d were used for samplings. Equilibration of the system was monitored by the redox potential. On the first day of each run, ruminal fluid and solid digesta were obtained from 2 out of the 3 nonlactating rumen-cannulated Brown Swiss cows kept at the Clinic for Ruminants at the University of Veterinary Medicine (Vienna, Austria) at about 3 h after morning feeding. Only 1 donor cow was available for the second experimental run. The donor cows had been fed with hay *ad libitum* and a daily allowance of 0.5 kg of commercial concentrates (KuhKorn PLUS Energie, Garant-Tiernahrung GmbH, Pöchlarn, Austria). The cows were kept according to Austrian guidelines for animal welfare. Before use, ruminal fluid of the cows was mixed and filtered through 4 layers of medical gauze (1 mm pore size). Each fermenter was inoculated with 600 mL of strained ruminal fluid and 100 mL of artificial saliva. Subsequently, a pair of nylon bags (120 × 65 mm, 150 µm pore size, Fa. Linker Industrie-Technik GmbH, Kassel, Germany) was added to each fermenter, one filled with the experimental diet and another bag filled with solid ruminal digesta. On the second day, for each fermenter the digesta bag was

Table 1. Chemical composition of diet ingredients

Item	Hay	Barley grain	Soybean meal	DDGS ¹	DDGS + 1% GSM ²	DDGS + 5% GSM	DDGS + 10% GSM	DDGS + 20% GSM
Chemical composition (% of DM, unless otherwise noted)								
DM (%)	83.90	88.40	91.90	90.90	90.20	90.70	90.70	90.90
OM	90.59	97.14	93.12	92.43	92.29	92.45	92.75	93.15
CP	11.35	12.26	49.07	36.13	35.8	34.88	34.76	31.35
NDF	57.3	21.1	19.7	37.8	39.5	38.4	41.8	45.9
NFC ³	20.09	60.99	21.62	11.63	10.18	12.36	9.79	10.27
Ether extract	1.89	2.78	2.77	6.84	6.85	6.77	6.44	5.63
Phenolic compounds (% of DM)								
Total phenols	1.95	0.79	0.75	1.50	1.55	1.74	2.14	2.78
Free phenols	1.16	0.52	0.66	1.40	1.45	1.44	1.37	1.35
Tannins	0.79	0.27	0.09	0.10	0.10	0.30	0.77	1.43
Nontannins	1.16	0.52	0.66	1.40	1.45	1.44	1.37	1.35

¹DDGS = dried distillers grains plus solubles.

²DDGS fortified with grape seed meal (GSM).

³NFC = 100 - (ash + CP + NDF + ether extract).

replaced with a fresh feed bag containing the respective experimental diet. During incubation, artificial saliva was continuously infused at a rate of 326 ± 19.2 mL/d ($\sim 2\%/h$) using a 12-channel peristaltic pump (model ISM932, Ismatec, IDEX Health & Science GmbH, Wertheim, Germany). Effluent and fermentation gases were collected daily in effluent bottles kept in an ice tub and gas-tight bags (TecoBag 8 L, Tesseroux Container GmbH, Bürstadt, Germany), respectively. On daily basis, each feed bag, which was incubated for 48 h, was withdrawn, rinsed and squeezed above the fermenter with 50 mL of prewarmed buffer, and then replaced with a new feed bag. Subsequently, each fermenter was tightly closed and flushed with nitrogen gas for 3 min to reestablish anaerobic conditions. On the sampling days, 1 h before feed bag replacement, 15 mL of incubation fluid was sampled directly from each fermenter through a 3-way valve. About 12 mL of the liquid sample was used for daily measurement of fermentation characteristics, 2 mL was immediately stored at -20°C for short-chain fatty acid (SCFA) analysis, and another 1 mL was snap frozen in liquid nitrogen and stored at -80°C until DNA extraction. The incubated feed (residue) bags were hand-washed with running cold water until the water was clear and kept at -20°C for chemical composition analysis.

Daily Measurements of Fermentation Characteristics

Redox potential, pH, and ammonia concentration of incubation fluid samples were immediately determined using a pH meter (Seven Multi TM, Mettler-Toledo GmbH, Schwerzenbach, Switzerland) equipped with the respective electrodes. Volume of effluent fluid was recorded to control the optimal buffer infusion. Daily gas volume was measured by the water replacement

method (Klevenhusen et al., 2014) and gas composition was determined using an infrared detector (ATEX Biogas monitor Check BM 2000, Ansyco, Karlsruhe, Germany).

Concentration and composition of SCFA (acetate, propionate, isobutyrate, *n*-butyrate, isovalerate, and *n*-valerate) were analyzed by GC, as described previously (Klevenhusen et al., 2014). Briefly, incubation fluid samples were thawed and then centrifuged at $20,000 \times g$ for 20 min at 20°C . The supernatant (0.8 mL) was transferred into a fresh tube with 0.2 mL of oxalic acid dehydrate and 0.2 mL of the internal standard (4-methyl valeric acid). The mixture was centrifuged again to remove remaining precipitated materials. The clear supernatant was analyzed for SCFA via GC (Fisons GC model 8060 MS DPFC, No.: 950713, Rodena, Italy) equipped with a flame-ionization detector and a $15\text{-m} \times 0.530\text{-mm}$ capillary column (SN US46185178, JW Scientific, Folsom, CA). Temperatures of injector and detector were 170 and 190°C , respectively. Helium was used as carrier gas with flow rate of 1 mL/min. Identification of the peaks was performed using Stratos Software (Stratos version 4.5.0.0, Polymer Laboratories, Shropshire, UK).

Chemical Composition Analysis

Chemical composition of feed and feed residues samples were analyzed by the proximate nutrient analysis. Prior to analysis, feed residue samples were pooled across the last 5 d of each experimental run. Experimental feeds and the pooled residue samples were oven-dried at 50°C for 48 h, ground passing through a 0.75-mm sieve. The ground materials were randomly sampled for determination of DM, OM, CP, crude fat, and NDF. Dry matter was analyzed by oven drying at

100°C overnight and ash by combustion of samples at 580°C overnight. Organic matter was then calculated from the ash content. Crude protein was analyzed by the Kjeldahl method (VDLUFA, 2007). The content of NDF was determined with the Fiber Therm FT 12 (Gerhardt GmbH & Co. KG, Königswinter, Germany) using heat-stable α -amylase. The NDF values are reported exclusive of residual ash. Crude fat was analyzed as ether extract using a Soxhlet extractor (Extraction System B-811, Büchi, Flawil, Switzerland). Subsequently, apparent nutrient disappearance, termed as nutrient degradation, was calculated from the difference between the amount found in diet and amount recovered in feed residue.

Analysis of Phenolic Compounds

All diet ingredients, and additionally pure GSM (red and white) samples, were finely ground with a ball mill for analysis of phenols (i.e., total phenols, tannin, and nontannin phenols). Briefly, to distinguish between free and bound total phenols, 2 extraction techniques were employed. For extraction of free phenols, about 400 mg of each ground sample was mixed with 18 mL of 60% ethanol (vol/vol) and 2.5 mL of distilled water in a 25-mL test tube. The test tube was placed in an ultrasonic bath at room temperature for 1 h. Subsequently, the mixture volume was made up to 25 mL with 60% ethanol (vol/vol) before filtrating through a cellulose paper. The clear extract was then used for free phenol content determination using the Folin-Ciocalteu method. For total phenol extraction (free plus bound forms), instead of distilled water 2.5 mL of 2N HCl was used and the acid hydrolysis underwent at 90 to 95°C for 2 h in a water bath. After cooling, the mixture volume was made up to 25 mL with 60% ethanol (vol/vol) before the filtration the same as the extraction of free phenols. For determination of tannins, 100 mg of polyvinyl-polyrrolidone was added to 1 mL of ethanolic or acidic extract diluted with 1 mL of distilled water, well mixed, and incubated for 15 min at 4°C to precipitate tannins. After centrifugation the clear supernatant (nontannins) was used for the phenolic assay.

The phenolics in the extract or after precipitation were determined by the Folin-Ciocalteu method in which the colorimetric reactions were adapted to be measured with a microplate absorbance reader (iMark, Bio Rad Laboratories, Inc., Hercules, CA). In the wells of the microplate, 10- μ L extracts were added to 100 μ L of distilled water followed by 5 μ L of Folin-Ciocalteu reagent, 10 μ L of Na_2CO_3 (35% in distilled water), and 125 μ L of distilled water. After 1 h of resting in the dark, the absorbance was measured at 750 nm. The phenolics were expressed as gallic acid equivalents.

Genomic DNA Extraction and Quantitative PCR

Prior to DNA extraction, the frozen incubation fluid samples from each fermenter each run were thawed at 4°C, pooled, and mixed well (total $n = 36$). Then DNA was extracted from about 250 μ L of homogenized sample using the PowerSoil DNA extraction kit with a bead beating method (MoBio Laboratories Inc., Carlsbad, CA). Primers targeting the various microbial genera and species were selected based on the recent studies on rumen microbiota (Table A1). Quantification of DNA in samples was done on a Stratagene Mx3000P QPCR System (Agilent Technologies, Santa Clara, CA) using Brilliant II SYBR Green QPCR Low ROX master mix (Agilent Technologies) mixed with the selected primer set at a concentration of 400 nmol for each primer and 1 μ L of genomic DNA. Each amplification reaction was run in duplicate with a final volume of 25 μ L. Amplification procedure and subsequent gel electrophoresis procedure for checking PCR products were previously described in Metzler-Zebeli et al. (2013). Standard curves were constructed using the universal primer set 27F-1492R for quantification of total bacterial 16S rRNA gene, primer set P.SSU.-54F-P.SSu-1747r for total protozoal 18S rRNA gene, and primer set 109F-934R for archaeal 16 rRNA gene. Amplification efficiency and quantification of final copy numbers (gene copies per milliliter of incubation fluid) were calculated (Metzler-Zebeli et al., 2013). Amplification efficiency was calculated according to the equation $E = 10^{(-1/\text{slope})} - 1$ (Table A1). Gene copy numbers of total bacteria, total protozoa, total methanogens, and fungi were determined by relating the C_q (quantification cycle) values to standard curves. The final copy numbers per milliliter of incubation fluid of total bacteria, total protozoa, total methanogens, and fungi were calculated using the equation $(QM \times C \times DV) / (S \times V)$, where QM was the quantitative mean of the copy number, C was the DNA concentration of each sample, DV was the dilution volume of extracted DNA, S was the DNA amount (ng) subjected to analysis, and V was the weight of the sample (g) subjected to DNA extraction (Li et al., 2012a).

The relative abundance of target bacterial, protozoal, and archaeal group or species was expressed as a proportion of total bacterial 16S rRNA gene, protozoal 18S rRNA gene, and archaeal 16S rRNA gene, respectively, calculated from threshold cycle (C_t) value [relative quantification = $2^{-(C_t \text{ target} - C_t \text{ total number})}$] (Metzler-Zebeli et al., 2013). In addition to protein degradation, hydrolysis of urea also contributes to ammonia pool in the rumen; therefore, we investigated the abundance of the urease gene. Relative quantification of the urease gene levels was calculated using the $\Delta\Delta C_t$ method (Livak

and Schmittgen, 2001). The total bacterial 16S rRNA gene was used for normalization.

Statistical Analyses

All statistical analyses were performed using SAS (version 9.2, SAS institute Inc., Cary, NC), and only linear discriminant analysis (LDA) was carried out using JMP software (version 10, SAS Institute Inc., Cary, NC). Data of daily measurements were analyzed as repeated measures. Fermenter was considered as the experimental unit on which all the measures were repeated (sampling days). The model consisted of the fixed effects of dietary treatment and sampling day, considering the variation between experimental runs and the covariation within experimental units with a compound symmetry covariance structure, which was specified by the RANDOM statement and the REPEATED statement, respectively (Littell et al., 1998). For degradation and quantitative PCR data, as samples were pooled across sampling days before laboratory analysis, means of analytical replicates were analyzed as complete randomized design with the statistical model consisting of the fixed effect of dietary treatment and the random effect of experimental run. Some extreme values of ammonia and fermentation gas were noted. These values were considered outliers when their studentized residual was beyond ± 3.0 (Khiaosa-ard and Zebeli, 2013) and were eliminated from the data set. Other gas composition data of these outlier values were excluded accordingly. The ANOVA was carried out using the MIXED procedure of SAS. Pairwise comparison among least squares means of control and other treatments containing DDGS, as well as among DDGS diet and treatments with GSM, followed Tukey's method. Orthogonal contrasts were performed to test linear and quadratic effects of GSM fortification level.

The LDA was used to examine potential relationships between dietary treatments and gene copies of microbial groups in incubation fluid. The LDA shows similarities and differences regarding microbial variables among the data sets (i.e., diets) in a way that the diets exhibiting similarities are clustered together and those that are different are placed further apart in the canonical axes 1 and 2. Inter-relationships among variables (microbial gene copy numbers and averaged fermentation traits) were determined with principal component analysis (PCA) using the Princomp procedure of SAS, which is based on variables standardized to zero mean and unit variance. The correlation matrix was used to generate principal component eigenvalues and associated loading plots. This procedure is necessary when variables are measured in different units (Quinn and Keough, 2001). Proc Corr of SAS was also used to compute the correla-

tions between variables and principal components and the significance level of the variables.

RESULTS

Fermentation Characteristics

Results of fermentation characteristics are shown in Table 2. Dietary treatment did not affect pH of incubation fluid ($P = 0.940$) with average values of 6.6 to 6.7 across treatments. Control diet had greatest redox potential ($P < 0.001$), but lowest ammonia concentration ($P = 0.024$). Both redox potential and ammonia concentration did not largely change with fortification of GSM compared with pure DDGS. Concentrations of carbon dioxide and methane in fermentation gas (%) were quite low resulting from a dilution of nitrogen gas used for flushing the headspaces of the fermenters during feed bag exchange process. All methane variables, concentration of methane in fermentation gas (%), absolute methane formation (mL/d), and the formation per gram of degraded OM or NDF were significantly altered by GSM fortification of DDGS ($P < 0.01$ for all except degraded OM, $P = 0.015$), and the inhibitory effect was more evident when the fortification level was $\geq 5\%$. The DDGS had a slightly lower value (5–10%) than most methane variables compared with that of control ($P > 0.05$), but DDGS decreased methane per milliliter of degraded NDF by 36% ($P < 0.001$). When comparing GSM fortification with control diet, the GSM treatments decreased these methane variables by 19 to 23%; for methane per milliliter of degraded NDF a 29 to 51% reduction was seen.

Dietary treatment did not alter total SCFA formation, with a range of 94.3 to 99.1 mM ($P = 0.731$); however, the SCFA profile differed among treatments (Table 2). Acetate proportion of DDGS treatment was similar to the value of control. However, this variable linearly increased with increasing GSM fortification levels ($P = 0.037$). The maximum change occurred with treatment at 20% GSM fortification in which acetate proportion was about 3.7% higher than the control. Propionate proportion remained unaffected, although a slight trend for a quadratic effect of GSM fortification level was noted ($P = 0.072$). Acetate-to-propionate ratio was similar among treatments, ranging from 1.35 to 1.46. Butyrate and isovalerate proportions were decreased with DDGS treatment compared with control ($P < 0.01$). Butyrate was further decreased with increasing GSM fortification levels (linear effect, $P = 0.003$). Isobutyrate proportion was greater ($P < 0.001$) in the DDGS treatment than in control diet, but GSM fortification did not further affect this variable. A quadratic effect of GSM fortification level on valer-

Table 2. Rumen fermentation characteristics and nutrient degradability of supplied feed (apparent disappearance after 48-h incubation) as affected by dietary treatment

Item	Level of GSM in DDGS ² (%)						SEM	Contrast		
	Control ¹	0	1	5	10	20		Con vs. DDGS ³	GSM Lin ⁴	GSM Quad ⁵
pH	6.65	6.64	6.65	6.65	6.64	6.64	0.043	0.532	0.807	0.429
Redox (mV)	-217.7 ^a	-238.7 ^b	-242.8 ^b	-248.3 ^b	-244.6 ^b	-241.4 ^b	6.14	<0.001	0.384	0.014
Ammonia (mM)	18.8	22.4	18.7	19.6	22.5	20.0	3.97	0.024	0.791	0.297
Carbon dioxide (%)	19.8 ^a	18.7 ^{ab}	19.4 ^{ab}	17.1 ^{bc}	16.1 ^c	16.4 ^c	0.57	0.154	<0.001	0.885
Methane (%)	2.01 ^a	1.87 ^{ab}	1.90 ^{ab}	1.63 ^b	1.54 ^b	1.61 ^b	0.091	0.268	0.002	0.442
Methane (mL/d)	29.5 ^a	27.7 ^{ab}	28.6 ^{ab}	24.1 ^{ab}	22.6 ^b	23.4 ^{ab}	1.594	0.405	0.004	0.668
Methane (mL/g of digestible OM)	5.04 ^a	4.61 ^{ab}	4.88 ^{ab}	4.12 ^{ab}	3.89 ^b	4.09 ^{ab}	0.283	0.249	0.015	0.727
Methane (mL/g of digestible NDF)	34.0 ^a	21.7 ^{bc}	24.0 ^b	20.1 ^{bc}	17.6 ^c	16.4 ^c	2.80	<0.001	<0.001	0.314
Short-chain FA (mM)	96.7	99.1	94.3	98.1	95.2	98.6	6.22	0.511	0.978	0.324
Individual (mol/100 mol)										
Acetate	48.8 ^b	49.2 ^{ab}	50.0 ^{ab}	49.9 ^{ab}	50.0 ^{ab}	50.6 ^a	0.76	0.441	0.037	0.995
Propionate	36.3	36.0	35.6	35.3	35.1	36.0	1.13	0.477	0.807	0.072
Butyrate	8.14 ^a	7.55 ^b	7.55 ^b	7.24 ^b	7.13 ^b	7.21 ^b	0.347	<0.001	0.003	0.428
Isobutyrate	0.702 ^b	0.802 ^a	0.762 ^a	0.775 ^a	0.785 ^a	0.760 ^{ab}	0.0386	<0.001	0.192	0.620
Valerate	3.72 ^{bc}	4.29 ^{ab}	3.97 ^b	4.56 ^a	4.82 ^a	3.25 ^c	0.667	0.023	0.027	<0.001
Isovalerate	2.33 ^a	2.17 ^b	2.16 ^b	2.20 ^{ab}	2.14 ^b	2.14 ^b	0.432	0.004	0.575	0.590
Acetate:propionate	1.35	1.37	1.46	1.42	1.43	1.41	0.064	0.679	0.720	0.267
Degradation (%)										
DM	54.0 ^{ab}	55.4 ^a	54.3 ^{ab}	54.2 ^{ab}	53.9 ^{ab}	53.0 ^b	0.53	0.025	<0.001	0.938
OM	52.4 ^{ab}	53.7 ^a	52.6 ^{ab}	52.6 ^{ab}	52.2 ^{ab}	51.1 ^b	0.53	0.047	<0.001	0.806
CP	50.2 ^b	60.0 ^a	59.4 ^a	60.5 ^a	58.9 ^a	59.5 ^a	1.05	<0.001	0.642	0.913
NDF	20.6	26.0	24.2	24.1	24.4	26.0	2.29	0.017	0.953	0.208

^{a-c}Least squares means sharing no common superscripts are significantly different ($P < 0.05$) according to Tukey's method.

¹Control diet contained soybean meal as the protein source (Table 1).

²DDGS = dried distillers grains plus solubles; GSM = grape seed meal.

³Control vs. DDGS (0% GSM).

⁴Linear response of grape seed meal levels (including 0%) fortified in DDGS.

⁵Quadratic response of grape seed meal levels (including 0%) fortified in DDGS.

ate proportion ($P < 0.001$) was observed, and the 10% GSM treatment resulted in the highest value.

Nutrient Degradation

Degradation of nutrients, especially CP ($P < 0.001$), was affected by dietary treatment (Table 2). Degradation of DM and OM in the control diet was lower than in DDGS ($P < 0.05$). Fortification level of GSM linearly decreased the degradation of both DM and OM ($P < 0.001$). The DDGS in the diet increased degradation of CP by 20% compared with control ($P < 0.001$). This was also true in GSM-fortification treatments with higher CP degradation (17.3–20.5%) than the control. We detected no linear or quadratic effect of GSM fortification on CP degradation. The degradation of NDF in DDGS treatment was greater than the control diet ($P = 0.017$), whereas GSM fortification did not affect this variable.

Ruminal Microbiota

Dietary treatment altered both the abundance and the composition of ruminal microbes (Table 3), but less for the abundance in the expression of urease gene (Figure 1). Total bacterial gene copy numbers were linearly decreased at increasing fortification levels of GSM ($P = 0.042$). Among the bacterial groups studied, genus *Prevotella* was the most abundant bacterial group investigated and its proportion was significantly affected by treatment. Interestingly, the proportions of genus *Prevotella* ($P = 0.002$) and *Clostridium* cluster XIVa ($P = 0.02$) were enhanced by the presence of DDGS without any associative effect of GSM fortification. Proportions of cellulolytic bacterium *Ruminococcus albus* tended to increase by DDGS treatment ($P = 0.094$) compared with soybean meal-based control diet, but GSM fortification did not affect this bacterium. A quadratic effect of GSM fortification of the DDGS was observed on *Fibrobacter succinogenes* ($P = 0.018$) and *Lactobacillus* spp. ($P = 0.047$). There was no treatment effect on the remaining bacterial groups or species (*Enterobacteriaceae*, *Clostridium* cluster I and IV, *Ruminococcus flavefaciens*, and *Butyrivibrio fibrisolvens*). The coverage of the investigated bacterial groups and species as percentage of total bacteria in control was lower than in DDGS treatment ($P = 0.009$), but the GSM fortification of DDGS did not further affect this variable. For methanogenic archaea, their abundance was similar but their composition differed among treatments. Whereas the *Methanosphaera* spp. proportion remained unchanged between treatments ($P = 0.562$), *Methanobrevibacter* spp. proportion was highest in the control diet. The DDGS treatment decreased the pro-

portion by 28% (contrast analysis, $P = 0.037$) compared with control, whereas GSM fortification levels showed a quadratic effect ($P = 0.011$). These 2 methanogenic genera with clear predominance of *Methanobrevibacter* covered more than half of the total methanogens (57.3–68.4%), except for 20% GSM fortification of DDGS in which its coverage was only 40%, significantly lower than control ($P < 0.05$). The DDGS decreased the total coverage from these 2 genera compared with control ($P = 0.038$) and a quadratic effect of GSM fortification was seen ($P = 0.012$). Protozoa numbers were affected by GSM fortification level, which linearly decreased their abundance ($P = 0.007$). However, proportion of the most abundant species *Entodinium* spp. remained similar between treatments. Last, gene copy numbers of total anaerobic fungi were lower in DDGS and DDGS with 20% GSM compared with control ($P < 0.05$), whereas other GSM treatments showed intermediate values. As shown in Figure 1, the urease gene tended to be more abundant with the DDGS diet containing 5% GSM than the control diet ($P = 0.07$) or DDGS with lower GSM levels ($P < 0.10$).

Multivariate Analysis

Linear discriminant analysis of dietary treatments and microbial abundance in incubation fluid revealed 4 clusters for the effects of dietary treatments. Unfortified DDGS and the 5 and 20% GSM fortification treatments were clustered together, whereas the control diet and the other GSM treatments were clustered further apart from each other (Figure 2). This means that the microbiota of the 3 treatments that clustered together had a higher similarity, whereas the control diet largely differed from the other treatments and the abundances of *B. fibrisolvens* and *Enterobacteriaceae* discriminated the control from the others. The DDGS treatment was more correlated with *F. succinogenes* and *Clostridium* cluster I, whereas total fungi and *Lactobacillus* spp. discriminated best for the treatment with 1% GSM fortification. The treatment with 10% GSM fortification was more correlated with the 2 methanogenic species than any other treatment.

A loading plot originating from PCA is a plot of the relationship between original variables and subspace dimension. The loading plots revealed relationships among variables studied (Figure 3). Only the first 3 components were plotted and in total they explained 58.5% of the total variation (26.2, 20.0, and 12.3%, respectively). Propionate proportion ($r = 0.627$, $P < 0.001$) and total SCFA ($r = 0.670$, $P < 0.001$) correlated strongly with the first component, whereas isovalerate proportion had the negative relationship ($r = -0.863$, $P < 0.001$; Figure 3a) with the first component. This component

Table 3. Gene copy numbers (\log_{10} gene copies/mL) and relative proportion of ruminal microbes of 48-h rumen incubation fluid as affected by dietary treatment

Item	Level of GSM in DDGS ² (%)						SEM	Contrast		
	Control ¹	0	1	5	10	20		Con vs. DDGS ³	GSM Lin ⁴	GSM Quad ⁵
Total bacteria	9.19	9.12	9.14	9.18	9.07	9.02	0.066	0.242	0.042	0.057
Proportion ⁶ (%)										
<i>Prevotella</i>	6.28 ^b	8.96 ^a	8.90 ^a	8.63 ^{ab}	8.83 ^a	8.86 ^a	0.558	0.002	0.861	0.779
<i>Enterobacteriaceae</i> ($\times 10^{-3}$)	2.34	4.20	5.01	2.85	2.40	3.80	0.988	0.182	0.270	0.431
<i>Fibrobacter succinogenes</i>	0.215	0.247	0.238	0.217	0.227	0.287	0.270	0.213	0.241	0.018
<i>Clostridium</i> cluster I ($\times 10^{-6}$)	5.83	7.96	9.82	13.99	17.92	11.16	6.319	0.807	0.480	0.466
<i>Clostridium</i> cluster IV	1.44	1.42	1.41	1.39	1.32	1.31	0.143	0.868	0.307	0.914
<i>Ruminococcus albus</i> ($\times 10^{-2}$)	1.61	2.14	2.04	2.50	2.35	2.29	0.690	0.094	0.375	0.516
<i>Ruminococcus flavefaciens</i> ($\times 10^{-5}$)	1.26	1.71	2.74	2.39	0.754	1.75	0.696	0.548	0.291	0.526
<i>Clostridium</i> cluster XIVa	0.92	1.14	1.11	1.16	1.02	1.11	0.105	0.020	0.462	0.865
<i>Butyrivibrio fibrisolvens</i> ($\times 10^{-3}$)	1.46	1.02	1.02	0.96	0.82	1.12	0.229	0.126	0.979	0.484
<i>Lactobacillus</i> group	1.52	1.45	1.65	2.12	2.13	1.74	0.322	0.821	0.123	0.047
Coverage (%)	10.38 ^b	13.22 ^{ab}	13.36 ^{ab}	13.52 ^a	13.53 ^a	13.31 ^{ab}	0.794	0.009	0.873	0.747
Total methanogenic archaea	6.33	6.66	6.43	6.63	6.50	6.61	0.119	0.763	0.172	0.477
Proportion ⁶ (%)										
<i>Methanobrevibacter</i> spp.	67.9 ^a	49.0 ^{ab}	58.7 ^{ab}	61.7 ^{ab}	56.7 ^{ab}	39.5 ^b	15.52	0.037	0.283	0.011
<i>Methanosphaera</i> spp.	0.494	0.517	0.391	0.394	0.540	0.493	0.1516	0.820	0.659	0.268
Coverage (%)	68.4 ^a	49.6 ^{ab}	59.1 ^{ab}	62.1 ^{ab}	57.3 ^{ab}	40.0 ^b	15.59	0.038	0.287	0.012
Total protozoa	6.36	6.23	6.36	6.30	5.77	5.71	0.179	0.622	0.007	0.214
Proportion ⁶ (%)										
<i>Entodinium</i> spp.	42.45	36.00	36.87	41.59	47.08	38.36	4.736	0.269	0.253	0.234
Total anaerobic fungi (%)	5.02 ^a	4.33 ^b	4.68 ^{ab}	4.57 ^{ab}	4.45 ^{ab}	4.28 ^b	0.396	0.003	0.520	0.079

^{a,b}Least squares means sharing no common superscripts are significantly different ($P < 0.05$) according to Tukey's method.

¹Control diet contained soybean meal as the protein source (Table 1).

²DDGS = dried distillers grains plus solubles; GSM = grape seed meal.

³Control vs. DDGS (0% grape seed meal).

⁴Linear response of grape seed meal levels (including 0%) fortified in DDGS.

⁵Quadratic response of grape seed meal levels (including 0%) fortified in DDGS.

⁶Relative quantification = $2^{-(Ct_{\text{target}} - Ct_{\text{total number}})}$. Ct = threshold cycle.

can be viewed as a measure of overall fermentation. Compared with other fermentation variables, methane variables, especially methane in milliliters per gram of degraded NDF ($r = 0.812$, $P < 0.001$) and OM ($r = 0.694$, $P < 0.001$) and butyrate proportion ($r = 0.680$, $P < 0.001$), had greater positive correlation with the second component, whereas ammonia production ($r = -0.737$, $P < 0.001$), acetate proportion ($r = -0.736$, $P < 0.001$), acetate to propionate ($r = -0.708$, $P < 0.001$), and NDF degradation ($r = -0.677$, $P < 0.001$) had a high negative correlation with this component. Methane and nutrient degradability variables are also the main contributors to the third component (Figure 3b). Thus, the last 2 components can be viewed as a measure of methane formation, which, however, was influenced by different factors.

The contribution of the microbial groups and species to ruminal fermentation can be interpreted from the position of variables on the loading plots as each variable represents its relationship to other variables. Variables clustering close to each other are strongly and positively correlated, whereas variables in the opposite direction are negatively correlated. Accordingly, methane vari-

ables were positively correlated with carbon dioxide percentage in total gas, butyrate, gene copy numbers of total fungi, and *R. flavefaciens* (Figure 3a), suggesting the major contribution of these microbes to the methane formation. Total methanogens, *Clostridium* cluster XIVa, *Prevotella*, and total SCFA were clustered together. These variables, and *R. albus*, oriented in an opposite direction to the cluster of methane variables. In addition, degradation of DM and OM, methane variables, and protozoa variables were clustered together and positively correlated with the third component (Figure 3b), reflecting the role of protozoa and the effect on nutrient degradability on methane variables, though with smaller magnitude as the third component explained smaller part of the total variation.

DISCUSSION

Effects of Unfortified DDGS on Ruminal Fermentation and the Role of Microbiota

In general, DDGS can vary considerably in nutrient composition as influenced by DDGS type (wet vs. dried), the type of original grains, and bioethanol plant

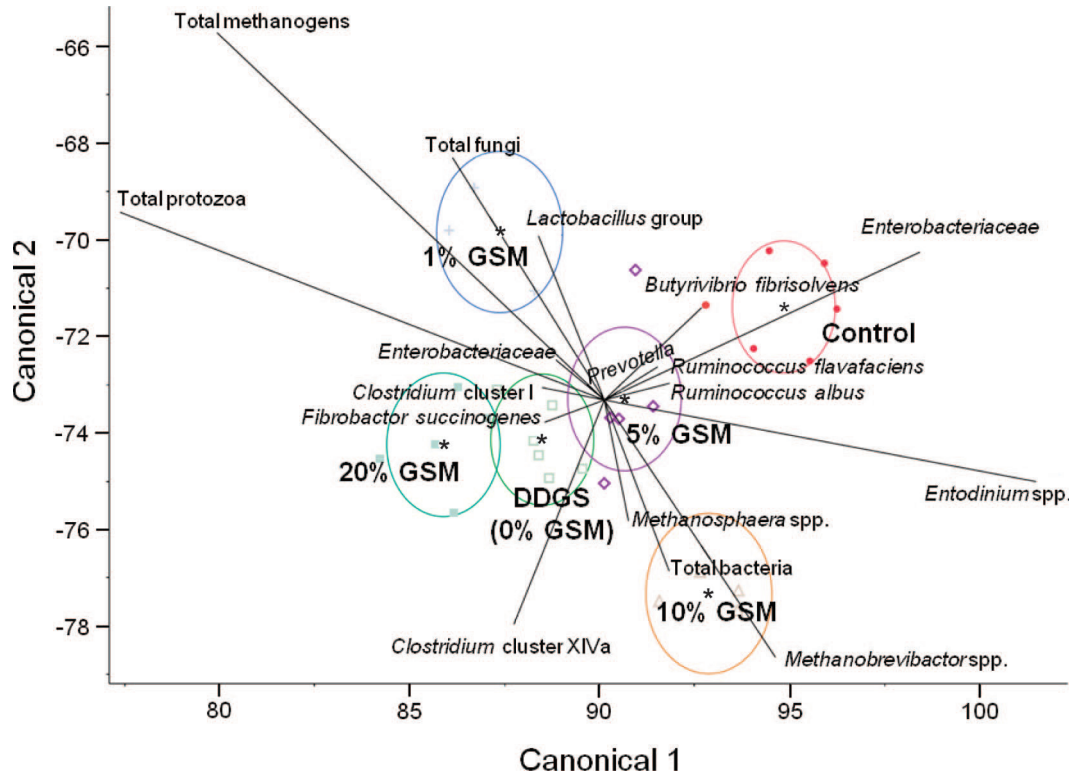


Figure 1. Linear discriminant analysis of the determinant gene copy numbers of microbial groups relative to dietary treatments: soybean meal-based control diet (●), and diets based on dried distillers grains plus solubles (DDGS) containing grape seed meal (GSM) at the 0 (□), 1 (+), 5 (◆), 10 (Δ), and 20% (■) level. The asterisk (*) in the middle of the circles indicates multivariate mean of each independent variable (i.e., diets), and the size of the circle indicates the 95% confidence limits of the mean. The distance between the diets in the canonical axis 1 and 2 reflects their dissimilarity. The straight lines are indicative of dependent variables included in the analysis, and their length and angles between them are function of the relative effects of independent variables. Color version available online.

origin (Nuez Ortín and Yu, 2009; Liu, 2012). In the present study, the nutritive value, based on the chemical composition, of unfortified DDGS was comparable to the general composition of DDGS reviewed by Liu (2012). We found that independent of GSM, DDGS inclusion decreased redox potential of the incubation fluid compared with control; however, all values were still in the normal range found in the rumen of cattle (Julien et al., 2010). Together with the maintained pH of about 6.7, this indicates optimal fermentation conditions for microbial growth and activity in the current Rusitec study.

Although total SCFA, acetate, and propionate were not affected, decreased butyrate and isovalerate and increased isobutyrate and valerate levels clearly showed that inclusion of DDGS modulated microbial metabolic activity when compared with the control treatment. Substituting DDGS for soybean meal and barley grain lowered the availability of easily degradable NFC, most likely starch, but increased the content of potentially degradable fiber. It is therefore possible that greater starch availability in the control diet compared with DDGS was associated with greater butyrate instead of

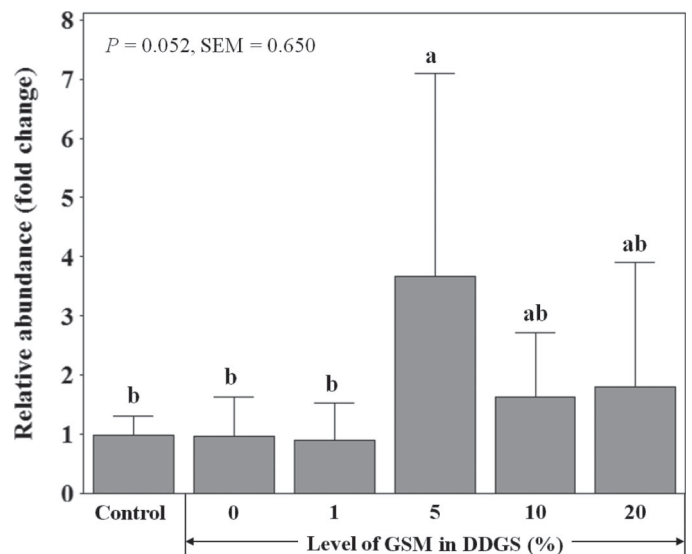


Figure 2. Relative abundance (expressed as fold change) of urease gene in response to dietary treatments: control = soybean meal-based control diet, and diets based on dried distillers grains plus solubles (DDGS) containing grape seed meal (GSM) at the 0, 1, 5, 10, and 20% level. Means carrying different letters (a,b) tend to differ ($P < 0.10$) according to Tukey's method.

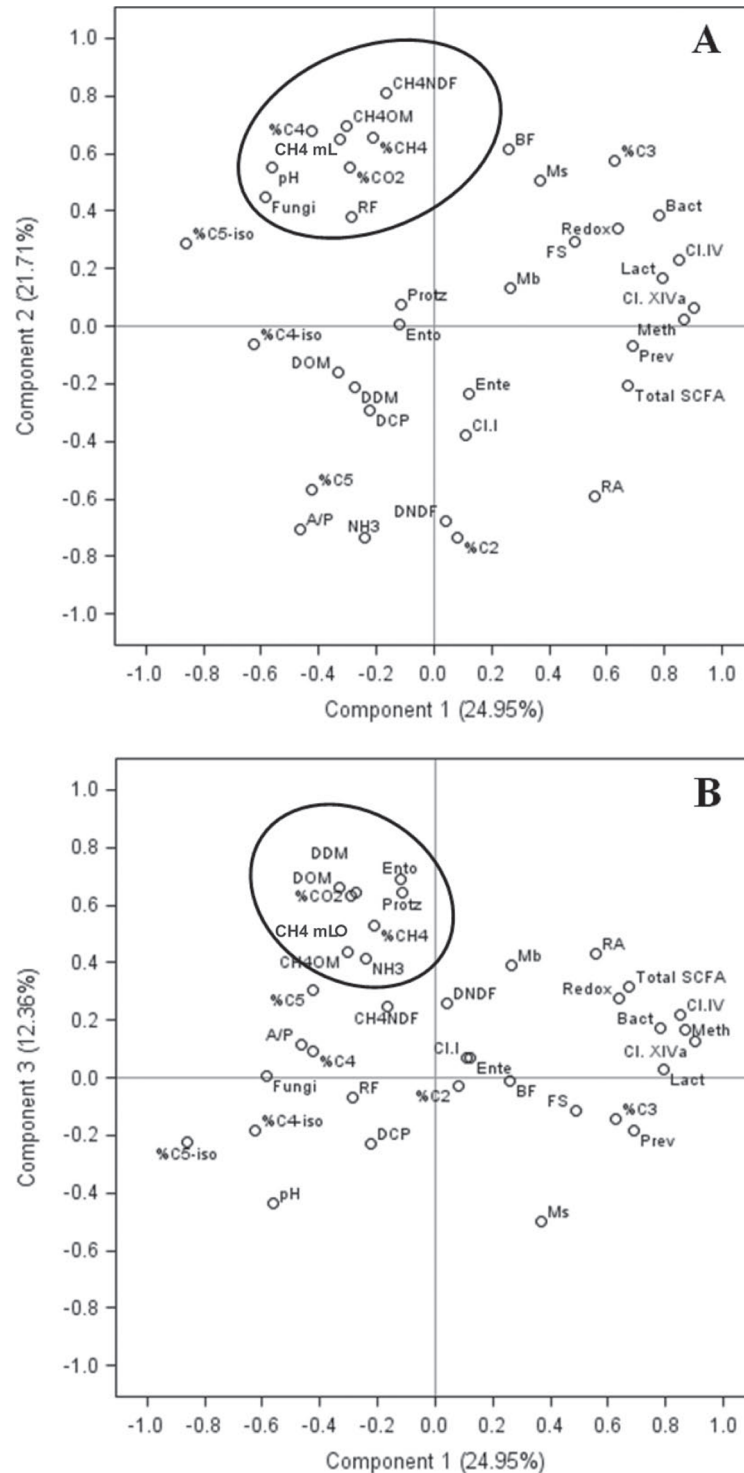


Figure 3. Loading plots of the first 3 principal components showing the relationships among variables; the ovals group variables that are positively associated with methane variables. A/P = acetate-to-propionate ratio; %CH₄ = methane percentage in total gas; CH₄ mL = methane per day (mL); CH₄NDF = methane formation per gram of degraded NDF; CH₄OM = methane formation per gram of degraded OM; %CO₂ = carbon dioxide percentage in total gas; %C₂ and %C₅ = molar percentage of acetate, propionate, butyrate, and valerate (%), respectively; %C₄- and %C₅-iso = molar percentage of isobutyrate and isovalerate, respectively; DCP, DDM, DNDF, and DOM = degraded CP, DM, NDF, and OM (%), respectively; NH₃ = ammonia (mM); Redox = redox potential (mV); Total SCFA = total short-chain FA (mmol/L); BF = *Butyrivibrio fibrisolvens*; Cl. I, IV, and XIVa = *Clostridium* cluster I, IV, and XIVa, respectively; Ente = *Enterobacteriaceae*; Ento = *Entodinium* spp.; Fungi = total fungi; FS = *Fibrobacter succinogenes*; Lact = *Lactobacillus* group; Mb = *Methanobrevibacter* spp.; Ms = *Methanosphaera* spp.; Meth = total methanogens; Prev = genus *Prevotella*; Protz = total protozoa; RA = *Ruminococcus albus*; RF = *Ruminococcus flavefaciens*.

the typical increase of propionate formation, as reported previously (Lettat et al., 2010). According to the SCFA profile, we expected a corresponding decrease in the abundance of butyrate-producing bacteria due to DDGS inclusion. Accordingly, an important ruminal butyrate producer, *B. fibrisolvens* (Pryde et al., 2002), tended to be less abundant with DDGS than with the control treatment. Interestingly, the proportion of *Clostridium* cluster XIVa, which also includes several butyrate-producing bacteria (Li et al., 2012b), was enhanced with DDGS compared with the control, indicating that other members—potentially fibrolytic members—than butyrate-producers were promoted. Promotion of fibrolytic bacteria by DDGS inclusion would be supported by higher abundance of *Prevotella* and *R. albus* with DDGS compared with the control.

One of the major environmental concerns about DDGS is an excess of ruminal protein supply, which can lead to an increase in N cycling and thus nitrogen excretion affecting ammonia and nitrous oxide emissions (McGinn et al., 2009; Hünerberg et al., 2013). Despite the similar CP contents among our diets, DDGS treatment still caused an increase in ammonia concentration compared with the soy-based control diet. This finding indicates higher amounts of RDP of DDGS than soybean meal and a lower utilization of the available ammonia by rumen microbiota due to lack of available energy.

A higher RDP of DDGS was clearly confirmed by higher CP degradation of the DDGS diet, and was supported by the elevated valerate and isobutyrate proportions, the end products of ruminal deamination of dietary AA. Most ruminal cellulolytic bacteria require valerate and branched-chain SCFA for growth (Zhang et al., 2013). Together with slightly decreased total bacteria abundance in the DDGS treatment, this reflects a lack of readily available energy (NFC) for bacterial protein synthesis. In our study, the replacement of soybean meal and some of barley grain by DDGS in the diet lowered the content of dietary NFC by 5%, and most likely that of starch, which is typically used as rapid energy source by rumen microbiota. Conversely, the substitution also increased the content of NDF, which might have led to a mismatch of energy and ammonia availability due to slower degradation of NDF than starch, and hence lower microbial protein synthesis and ammonia utilization. According to Li et al. (2012b), *Prevotella*, the most abundant genus in the rumen community, consists of a group of bacteria responsible for protein metabolism. Our relative quantification of microbiota also showed an increase of genus *Prevotella* in the bacterial community with unfortified DDGS. Similarly, Callaway et al. (2010) reported increased *Prevotella* in the rumen with increasing DDGS

levels in a basal feedlot diet. However, only the increase of *Prevotella* was not sufficient to entirely explain the current results of protein degradation. This was not surprising, as this genus is very large and a confounding effect from carbohydrate-degrading members was likely. Besides, other proteolytic bacteria that were not covered by the present primer sets used presumably contributed to the protein degradation.

We found that bacteria expressing the urease gene cannot fully describe the DDGS effect on ammonia enhancement, as the abundance found with unfortified DDGS was similar to that of the control, whereas only DDGS containing 5% GSM tended to elevate the abundance of this gene. This can be explained by the fact that urease gene represents a small proportion of total bacteria and the abundance of the urease gene may not entirely represent urease activity. The lack of influence was not surprising, as urea hydrolysis is carried out by bacteria adhering to ruminal epithelium (Huntington and Archibeque, 2000); perhaps for in vivo situations the effect of DDGS on urea hydrolysis may be more evident. Future DDGS-related research should pay particular attention to protein- and urea-degrading bacteria and metabolic activity of ammonia producers in the rumen.

Previous research has shown promising effects of DDGS on methane mitigation (McGinn et al., 2009). However, this was not evident herein, which was in agreement with Li et al. (2012c). Diet components such as NDF and fat have a profound effect on methane emissions in cattle by which NDF is more likely to promote the formation (Jayanegara et al., 2011), whereas fat has an opposite effect (Hünerberg et al., 2013). Surprisingly, compared with the control, we found similar methane formation in the DDGS treatment, which was accompanied by its higher degradability of nutrients including NDF. The increased NDF degradation was nevertheless expected because the replacement of DDGS increased NDF content (mostly of hemicellulolytic nature) of the diet and it is highly digestible (Nuez Ortín and Yu, 2009; Zhang et al., 2010a). According to Hünerberg et al. (2013), the methane mitigation effect of DDGS is likely to associate with the fat content of DDGS. They observed that high-fat corn DDGS and wheat DDGS plus oil decreased methane emissions in beef cattle, whereas without oil addition wheat DDGS showed no effect. This may explain the lack of a methane mitigation effect of the DDGS inclusion in the present experiment.

Associative Effects of GSM on Methanogenesis and the Role of Microbiota

In our study, total phenol content of the diet increased with increasing GSM fortification levels, but this was

more related to the increase of the tannin fraction than the nontannin fraction. Phenolic compounds, tannins in particular, have been shown to form complexes with protein and cellulose and alter composition and activity of several ruminal microbes; hence, they can decrease ruminal methane formation as well as ammonia production (Carulla et al., 2005; Jayanegara et al., 2010; Patra and Saxena, 2011). In agreement with these reports, increasing GSM fortification levels in DDGS, and thus the tannin levels, linearly decreased methane variables. Based on the microbiota and PCA results, the methane inhibition was associated with decreased abundance of fungi and bacteria rather than methanogens, decreased butyrate production, and, to a smaller extent, decreased nutrient degradation, most likely that of NFC.

With respect to microbial changes, despite the substantial decrease of methane by the GSM fortification, surprisingly the role of methanogens, both the abundance and the relative proportion of the 2 genera investigated, was not well recognized in our study. Nevertheless, we observed a quadratic effect of GSM on the proportion of *Methanobrevibacter* spp. As the predominant methanogenic species in the rumen, the contribution of *Methanobrevibacter* spp. on methane formation has been reported previously (Mohammed et al., 2011). Indeed, in several cases, not the methanogen populations but rather the diversity has been described as more important for enteric methane emissions (Mohammed et al., 2011; Khiaosa-ard and Zebeli, 2014). However, this needs to be looked at for specific species or strains levels (Khiaosa-ard and Zebeli, 2014), which was not the case in our study.

According to our PCA, total fungi, *R. flavefaciens*, and protozoa were associated with the methane results observed in the present study. These microbial variables clustered with methane variables, indicating that they varied together. Previous research showed that tannins resulted in ruminal methane suppression, which was often accompanied by a reduction in protozoa numbers (Khiaosa-ard et al., 2009; Jayanegara et al., 2010). The role of protozoa in methane formation is recognized as a large producer of hydrogen and the host of methanogens in the rumen (Mosoni et al., 2011). With respect to aforementioned unaltered methanogen populations, this indicates no strong association between protozoa and methanogen populations in the present study, which agrees with a previous report (Jayanegara et al., 2010). Therefore, the methane inhibition found here seemed to be a consequence of lower hydrogen supply for methanogenesis partly from protozoa inhibition.

To our knowledge, little information exists regarding direct effects of plant secondary compounds on ruminal anaerobic fungi and its contribution to methane forma-

tion. As in protozoa, anaerobic fungi have an organelle called hydrogenosome and produce hydrogen. Interspecies hydrogen transfer between anaerobic fungi and methanogens, thus influencing ruminal methanogenesis, has been documented (Bauchop and Mountfort, 1981; Mountfort et al., 1982). It can be expected that a decrease of anaerobic fungi will decrease methane. Supporting this assumption, we found that the presence of GSM tannin phenols inhibited the number of anaerobic fungi and, thus, might have lowered hydrogen available for methanogens. Tannins can be toxic to fungi and yeast (Scalbert, 1991) and decrease their fiber-degrading ability (Patra and Saxena, 2011). Several mechanisms describing antimicrobial properties of tannins were proposed (Patra and Saxena (2011)). These mechanisms are related to its protein-binding property and its direct actions on microbial growth and metabolism.

Accumulated evidence demonstrates that the presence of different dominant cellulolytic bacterial species can influence methane production (Chaucheyras-Durand et al., 2010). The major cellulolytic bacteria in the rumen, *F. succinogenes*, *R. albus*, and *R. flavefaciens* (Koike and Kobayashi, 2009), were comparable among treatments in our study, but PCA indicated an association between methane formation and *R. flavefaciens* only. It is not clear why *R. flavefaciens* and *R. albus* did not cluster together and both even had different relationship with methane variables. Both ruminococci have similar carbohydrate fermentation and end products, only *R. flavefaciens* produces succinate as one of its major products (Krause et al., 1999). Also, these 2 bacterial species have been shown to form methane in cocultures with different methanogenic species (Latham and Wolin, 1977; Miller et al., 2000). Nevertheless, the different susceptibility of fibrolytic bacterial species to tannins has been recognized, which might result from different adhesion mechanisms to the substrates (Bento et al., 2005). It is plausible that *R. flavefaciens* were more susceptible to GSM tannin phenols than *R. albus*.

Methane formation accompanies the formation of acetate and butyrate. Our results demonstrate that only butyrate production was suppressed as GSM fortification levels increased. Considering carbohydrate metabolism of ruminal fungi and *R. flavefaciens* (Latham and Wolin, 1977; Bauchop and Mountfort, 1981), there should not be a concomitant effect on butyrate formation from these microorganisms. Hence, it can be assumed that the GSM inclusion possessed an inhibitory effect particularly on butyrate producing bacteria. One of the important fiber degraders and butyrate producers in the rumen, *B. fibrisolvens*, did show a trend toward lower abundance, although the relative

proportion was unaffected by GSM fortification. *Clostridium* cluster XIVa, which consists of many butyrate producers (Li et al., 2012c), tended to decrease with increasing GSM levels as well. As revealed by PCA, *B. fibrisolvens* was more correlated to butyrate and methane variables compared with *Clostridium* cluster XIVa, underlying the significance of this butyrate producer. Protozoa produce butyrate, too (Dehority, 2003), thus the suppression of protozoa may be partially responsible for the decreased butyrate formation due to GSM fortification. The present results also elicit a significant effect of butyrate producing pathway, over acetate, on methane formation.

Lastly, an associative effect of GSM on suppression of DM and OM degradability was observed in the current study, reflecting the binding property of GSM phenols. As revealed by PCA, the reduced degradability of DM and OM to some extent contributed to the decreased methane formation. As CP degradation was higher and NDF degradation was not affected by GSM fortification, the decrease in degradation of DM and OM was more likely to be related to carbohydrate fractions belonging to NFC (starch, sugars, and pectin). Opposite to our expectation, the results suggest that phenols of the GSM in our study had a greater affinity to NFC than to protein. It is known that phenolic compounds can bind to various carbohydrates, including pectin (Hanlin et al., 2010; Patra and Saxena, 2011). As NFC, such as sugar and pectin, have been shown to be a potential source of enteric methane formation (Hindrichsen et al., 2004, 2005), such complexes formed between GSM phenols and NFC would decrease ruminal degradation of these carbohydrates and, thus, lower methane formation. Hence, beyond the methane mitigating effect, GSM fortified in DDGS may be viewed as a beneficial ingredient for rumen health by lowering degradation of rapidly degradable carbohydrates, which helps to slow down a rapid production of SCFA.

The present study demonstrated the effects of DDGS and GSM on changes in microbiota investigated. Such changes were related to the modification of ruminal fermentation characteristics as explained and discussed previously. However, it is important to note that caution should be given when interpreting data from in vitro studies. In our study, the microbiota results were not accounted for the ruminal microorganisms attached to solid particles, which are also important for feed degradation. Likewise, contributions of rumen wall-associated bacteria are unlikely in vitro. These epithelial adherent bacteria, though less influenced by diet, are believed to have additional functions other than feed degradation, such as ruminal ammonia absorption (Li et al., 2012a). Therefore, a need exists for in vivo studies to validate our in vitro findings.

CONCLUSIONS

In conclusion, the inclusion of 19.5% of DDGS, as both protein and energy sources in a diet, only increased ruminal ammonia concentration due to its high CP degradation without other detrimental effects on ruminal fermentation. Fortification of GSM did have an associative effect on methane mitigation, which was connected with decreased numbers of *R. flavefaciens*, total fungi, and total protozoa, but to a lesser extent with archaea methanogens. The inhibition of these microbes could limit hydrogen supply for methanogenesis. Furthermore, a decrease was noted in ruminal DM and OM degradation by GSM fortification; however, this effect was not related to NDF, but NFC degradation. The results indicate that DDGS fortified with GSM can favorably modulate ruminal fermentation by lowering methane formation without adverse effect on fiber degradation. Also, it may be beneficial to rumen health and animal production by forming complexes with some rapidly degradable carbohydrates and thus might help to stabilize rumen pH in grain-rich diets.

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REFERENCES

- Abdelqader, M. M., A. P. Hippen, K. F. Kalscheur, D. J. Schingoethe, and A. D. Garcia. 2009. Isolipidic additions of fat from corn germ, corn distillers grains, or corn oil in dairy cow diets. *J. Dairy Sci.* 92:5523–5533.
- Baker, K. M., C. J. Gobler, and J. L. Collier. 2009. Urease gene sequences from algae and heterotrophic bacteria in axenic and nonaxenic phytoplankton cultures. *J. Phycol.* 45:625–634.
- Bartosch, S., A. Fite, G. T. Macfarlane, and M. E. McMurdo. 2004. Characterization of bacterial communities in feces from healthy elderly volunteers and hospitalized elderly patients by using real-time PCR and effects of antibiotic treatment on the fecal microbiota. *Appl. Environ. Microbiol.* 70:3575–3581.
- Bauchop, T., and D. O. Mountfort. 1981. Cellulose fermentation by a rumen anaerobic fungus in both the absence and the presence of rumen methanogens. *Appl. Environ. Microbiol.* 42:1103–1110.
- Bento, M. H. L., T. Acamovic, and H. P. S. Makkar. 2005. The influence of tannin, pectin and polyethylene glycol on attachment of

- 15N-labelled rumen microorganisms to cellulose. *Anim. Feed Sci. Technol.* 122:41–57.
- Callaway, T. R., S. E. Dowd, T. S. Edrington, R. C. Anderson, N. Bauer, P. J. Kononoff, and D. J. Nisbet. 2010. Evaluation of bacterial diversity in the rumen and feces of cattle fed different levels of dried distillers grains plus soluble using bacterial tag-encoded FLX amplicon pyrosequencing. *J. Anim. Sci.* 88:3977–3983.
- Carulla, J. E., M. Kreuzer, A. Machmüller, and D. Hess. 2005. Supplementation of *Acacia mearnsii* tannins decreases methanogenesis and urinary nitrogen in forage-fed sheep. *Aust. J. Agric. Res.* 56:961–970.
- Chaucheyras-Durand, F., S. Masséglia, G. Fonty, and E. Forano. 2010. Influence of the composition of the cellulolytic flora on the development of hydrogenotrophic microorganisms, hydrogen utilization, and methane production in the rumens of gnotobiotically reared lambs. *Appl. Environ. Microbiol.* 76:7931–7937.
- Dehority, B. A. 2003. *Rumen Microbiology*. Nottingham University Press, Nottingham, UK.
- Denman, S. E., and C. S. McSweeney. 2006. Development of a real-time PCR assay for monitoring anaerobic fungal and cellulolytic bacterial populations within the rumen. *FEMS Microbiol. Ecol.* 58:572–582.
- Goberna, M., M. Gadermaier, C. Garcia, B. Wett, and H. Insam. 2010. Adaptation of methanogenic communities to the cofermentation of cattle excreta and olive mill wastes at 37°C and 55°C. *Appl. Environ. Microbiol.* 76:6564–6571.
- Hanlin, R. L., M. Hrmova, J. F. Harbertson, and M. O. Downey. 2010. Condensed tannin and grape cell wall interactions and their impact on tannin extractability into wine. *Aust. J. Grape Wine Res.* 16:173–188.
- Hao, X., M. B. Benke, C. Li, F. J. Larney, K. A. Beauchemin, and T. A. McAllister. 2011. Nitrogen transformations and greenhouse gas emissions during composting of manure from cattle fed diets containing corn dried distillers grains with soluble and condensed tannin. *Anim. Feed Sci. Technol.* 166–167:539–549.
- Heilig, H. G. H. J., E. G. Zoetendal, E. E. Vaughan, P. Marteau, A. D. L. Akkermans, and W. M. De Vos. 2002. Molecular diversity of *Lactobacillus* spp. and other lactic acid bacteria in the human intestine as determined by specific amplification of 16S ribosomal DNA. *Appl. Environ. Microbiol.* 68:114–123.
- Hindrichsen, I. K., H. R. Wettstein, A. Machmüller, B. Jörg, and M. Kreuzer. 2005. Effect of the carbohydrate composition of feed concentrates on methane emission from dairy cows and their slurry. *Environ. Monit. Assess.* 107:329–350.
- Hindrichsen, I. K., H. R. Wettstein, A. Machmüller, C. R. Soliva, K. E. Bach Knudsen, J. Madsen, and M. Kreuzer. 2004. Effects of feed carbohydrates with contrasting properties on rumen fermentation and methane release in vitro. *Can. J. Anim. Sci.* 84:265–276.
- Hünerberg, M., S. M. McGinn, K. A. Beauchemin, E. K. Okine, O. M. Harstad, and T. A. McAllister. 2013. Effect of dried distillers grains plus solubles on enteric methane emissions and nitrogen excretion from growing beef cattle. *J. Anim. Sci.* 91:2846–2857.
- Huntington, G. B., and S. L. Archibeque. 2000. Practical aspects of urea and ammonia metabolism in ruminants. *J. Anim. Sci.* 77(E-Suppl.):1–11.
- Jayanegara, A., G. Goel, H. P. S. Makkar, and K. Becker. 2010. Reduction in methane emissions from ruminants by plant secondary metabolites: effects of polyphenols and saponins. Pages 151–157 in *Sustainable Improvement of Animal Production and Health*. N. E. Odongo, M. Garcia, and G. J. Viljoen, ed. Food and Agricultural Organization of the United Nations, Rome, Italy.
- Jayanegara, A., E. Wina, C. R. Soliva, S. Marquardt, M. Kreuzer, and F. Leiber. 2011. Dependence of forage quality and methanogenic potential of tropical plants on their phenolic fractions as determined by principal component analysis. *Anim. Feed Sci. Technol.* 163:231–243.
- Julien, C., J. P. Marden, C. Bonnefont, R. Moncoulon, E. Auclair, V. Monteils, and C. Bayourthe. 2010. Effects of varying proportions of concentrates on ruminal-reducing power and bacterial community structure in dry dairy cows fed hay-based diets. *Animal* 4:1641–1646.
- Khiaosa-ard, R., and Q. Zebeli. 2013. Meta-analysis of the effects of essential oils and their bioactive compounds on rumen fermentation characteristics and feed efficiency in ruminants. *J. Anim. Sci.* 91:1819–1830.
- Khiaosa-ard, R., and Q. Zebeli. 2014. Cattle's variation in rumen ecology and metabolism and its contributions to feed efficiency. *Livest. Sci.* 62:166–175.
- Khiaosa-ard, R., S. F. Bryner, M. R. L. Scheeder, H.-R. Wettstein, F. Leiber, M. Kreuzer, and C. R. Soliva. 2009. Evidence for the inhibition of the terminal step of ruminal α -linolenic acid biohydrogenation by condensed tannins. *J. Dairy Sci.* 92:177–188.
- Klevenhusen, F., K. Deckardt, Ö. Sizmaz, S. Wimmer, A. Muro-Reyes, R. Khiaosa-ard, R. Chizzola, and Q. Zebeli. 2014. Effect of black seed oil and *Ferula laeochytris* supplementation on ruminal fermentation as tested *in vitro* with the rumen simulation technique (Rusitec). *Anim. Prod. Sci.* <http://dx.doi.org/10.1071/AN13332>.
- Koike, S., and Y. Kobayashi. 2009. Fibrolytic rumen bacteria: Their ecology and functions. *Asian-Aust. J. Anim. Sci.* 22:131–138.
- Krause, D. O., B. P. Dalrymple, W. J. Smith, R. I. Mackie, and C. S. McSweeney. 1999. 16S rDNA sequencing of *Ruminococcus albus* and *Ruminococcus flavefaciens*: Design of a signature probe and its application in adult sheep. *Microbiology* 145:1797–1807.
- Latham, M. J., and M. J. Wolin. 1977. Fermentation of cellulose by *Ruminococcus flavefaciens* in the presence and absence of *Methanobacterium ruminantium*. *Appl. Environ. Microbiol.* 34:297–301.
- Lettat, A., P. Notzière, M. Silberberg, D. P. Morgavi, C. Berger, and C. Martin. 2010. Experimental feed induction of ruminal lactic, propionic and butyric acidosis in sheep. *J. Anim. Sci.* 88:3041–3046.
- Li, M., M. Zhou, E. Adamowicz, J. A. Basarab, and L. L. Guan. 2012a. Characterization of bovine ruminal epithelial bacterial communities using 16S rRNA sequencing, PCR-DGGE, and qRT-PCR analysis. *Vet. Microbiol.* 155:72–80.
- Li, R. W., S. Wu, R. L. Baldwin, W. Li, and C. Li. 2012b. Perturbation dynamics of the rumen microbiota in response to exogenous butyrate. *PLoS ONE* 7:e29392.
- Li, Y., M. He, C. Li, R. Forster, K. A. Beauchemin, and W. Yang. 2012c. Effects of wheat dried distillers' grains with soluble and cinnamaldehyde on *in vitro* fermentation and protein degradation using Rusitec technique. *Arch. Anim. Nutr.* 1:1–18.
- Littell, R. C., P. R. Henry, and C. B. Ammerman. 1998. Statistical analysis of repeated measures data using SAS procedure. *J. Anim. Sci.* 76:1216–1231.
- Livak, K. J., and T. D. Schmittgen. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta Ct}$ method. *Methods* 25:402–408.
- Liu, K. S. 2012. Chemical composition of DDGS. Pages 143–178 in *Distillers Grains, Product, Properties, and Utilization*. K. S. Liu and K. A. Rosentrater, ed. CRC Press, Boca Raton, FL.
- Loy, T. W., J. C. MacDonald, T. J. Klopfenstein, and G. E. Erickson. 2007. Effects of distillers grains or corn supplementation frequency on forage intake and digestibility. *J. Anim. Sci.* 85:2625–2630.
- Matsuki, T., K. Watanabe, J. Fujimoto, Y. Miyamoto, T. Takada, K. Matsumoto, H. Oyaizu, and R. Tanaka. 2002. Development of 16S rRNA-gene targeted group-specific primers for the detection and identification of predominant bacteria in human feces. *Appl. Environ. Microbiol.* 68:5445–5451.
- Matsuki, T., K. Watanabe, J. Fujimoto, T. Takada, and R. Tanaka. 2004. Use of 16S rRNA gene-targeted group-specific primers for real-time PCR analysis of predominant bacteria in human feces. *Appl. Environ. Microbiol.* 70:7220–7228.
- McGinn, S. M., Y.-H. Chung, K. A. Beauchemin, A. D. Iwaasa, and C. Grainger. 2009. Use of corn distillers' dried grains to reduce enteric methane loss from beef cattle. *Can. J. Anim. Sci.* 89:409–413.
- Metzler-Zebeli, B. U., S. Schmitz-Esser, F. Klevenhusen, L. Podstatzky-Lichtenstein, M. Wagner, and Q. Zebeli. 2013. Grain-rich diets differently alter ruminal and colonic abundance of microbial populations and lipopolysaccharide in goats. *Anaerobe* 20:65–73.
- Miller, T. L., E. Currenti, and M. J. Wolin. 2000. Anaerobic bioconversion of cellulose by *Ruminococcus albus*, *Methanobrevibacter*

- smithii*, and *Methanosarcina barkeri*. *Appl. Microbiol. Biotechnol.* 54:494–498.
- Mohammed, R., M. Zhou, K. M. Koenig, K. A. Beauchemin, and L. L. Guan. 2011. Evaluation of rumen methanogen diversity in cattle fed diets containing dry corn distillers grains and condensed tannins using PCR-DGGE and qRT-PCR analyses. *Anim. Feed Sci. Technol.* 166–167:122–131.
- Mosoni, P., C. Martin, E. Forano, and D. P. Morgavi. 2011. Long-term defaunation increases the abundance of cellulolytic ruminococci and methanogens but does not affect the bacterial and methanogen diversity in the rumen of sheep. *J. Anim. Sci.* 89:783–791.
- Mountfort, D. O., R. A. Asher, and T. Bauchop. 1982. Fermentation of cellulose to methane and carbon dioxide by a rumen anaerobic fungus in a triculture with *Methanobrevibacter* sp. Strain RA1 and *Methanosarcina barkeri*. *Appl. Environ. Microbiol.* 44:128–134.
- Muyzer, G., E. C. De Waal, and A. G. Uitterlinden. 1993. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. *Appl. Environ. Microbiol.* 59:695–700.
- Nuez Ortín, W. G., and P. Yu. 2009. Nutrient variation and availability of wheat DDGS, corn DDGS and blend DDGS from bioethanol plants. *J. Sci. Food Agric.* 89:1754–1761.
- Patra, A. K., and J. Saxena. 2011. Exploitation of dietary tannins to improve rumen metabolism and ruminant nutrition. *J. Sci. Food Agric.* 91:24–37.
- Penner, G. B., P. Yu, and D. A. Christensen. 2009. Effect of replacing forage or concentrate with wet or dry distillers' grains on the productivity and chewing activity of dairy cattle. *Anim. Feed Sci. Technol.* 153:1–10.
- Pryde, S. E., S. H. Duncan, G. L. Hold, C. S. Stewart, and H. J. Flint. 2002. The microbiology of butyrate formation in the human colon. *FEMS Microbiol. Lett.* 217:133–139.
- Quinn, G., and M. Keough. 2001. Principal components and correspondence analysis. Accessed May 5, 2014. <http://bio.classes.ucsc.edu/bio286/MicksBookPDFs/QK17.PDF>.
- Rinttilä, T., A. Kassinen, E. Malinen, L. Krogius, and A. Palva. 2004. Development of an extensive set of 16S rDNA-targeted primers for quantification of pathogenic and indigenous bacteria in faecal samples by real-time PCR. *J. Appl. Microbiol.* 97:1166–1177.
- Scalbert, A. 1991. Antimicrobial properties of tannins. *Phytochemistry* 30:3875–3883.
- Shi, J., J. Yu, J. Pohorly, J. C. Young, M. Bryan, and Y. Wu. 2003. Optimization of the extraction of polyphenols from grape seed meal by aqueous ethanol solution. *J. Food Agric. Environ.* 1:42–47.
- Skillman, L. C., A. F. Toovey, A. J. Williams, and A. D. G. Wright. 2006. Development and validation of a real-time PCR method to quantify rumen protozoa and examination of variability between *Entodinium* populations in sheep offered a hay-based diet. *Appl. Environ. Microbiol.* 72:200–206.
- Stevenson, D. M., and P. J. Weimer. 2007. Dominance of *Prevotella* and low abundance of classical ruminal bacterial species in the bovine rumen revealed by relative quantification real-time PCR. *Appl. Microbiol. Biotechnol.* 75:165–174.
- Sylvester, J. T., S. K. R. Kamati, Z. Yu, M. Morrison, and J. L. Firkins. 2004. Development of an assay to quantify rumen ciliate protozoal biomass in cows using real-time PCR. *J. Nutr.* 134:3378–3384.
- Tajima, K., R. I. Aminov, T. Nagamine, H. Matsuki, M. Nakamura, and Y. Benno. 2001. Diet-dependent shifts in the bacterial population of the rumen revealed with real-time PCR. *Appl. Environ. Microbiol.* 67:2766–2774.
- VDLUFA. 2007. Die Chemische Untersuchung von Futtermitteln. Handbuch der Landwirtschaftlichen Versuchs- und Untersuchungsmethodik. Band III. VDLUFA-Verlag, Darmstadt, Germany.
- Zebeli, Q., J. R. Aschenbach, M. Tafaj, J. Boguhn, B. N. Ametaj, and W. Drochner. 2012. Invited review: Role of physically effective fiber and estimation of dietary fiber adequacy in high-producing dairy cattle. *J. Dairy Sci.* 95:1041–1056.
- Zhang, H. L., Y. Chen, X. L. Xu, and Y. X. Yang. 2013. Effects of branched-chain amino acids on in vitro ruminal fermentation of wheat straw. *Asian-Aust. J. Anim. Sci.* 26:523–528.
- Zhang, S. Z., G. B. Penner, M. Abdelqader, and M. Oba. 2010a. Effects of feeding alfalfa hay on chewing, rumen pH, and milk fat concentration of dairy cows fed wheat distillers grains with soluble as a partial substitute of barley silage. *J. Dairy Sci.* 93:3243–3252.
- Zhang, S. Z., G. B. Penner, W. Z. Yang, and M. Oba. 2010b. Effects of partially replacing barley silage or barley grain with dried distillers grains with soluble on rumen fermentation and milk production of dairy lactating cows. *J. Dairy Sci.* 93:3231–3242.
- Zhou, M., E. Hernandez-Sanabria, and L. L. Guan. 2009. Assessment of the microbial ecology of ruminal methanogens in cattle with different feed efficiencies. *Appl. Environ. Microbiol.* 75:6524–6533.

APPENDIX

Table A1. Primers used for detection of ruminal microbes

Target	Primer sequence (5' → 3')		Amplicon size (bp)	A _T (°C)	Eff ¹ (%)	Ref ²
	Forward	Reverse				
Universal bacteria	CCTACGGGAGGCAGCAG	ATTACGGGGCTGCTGG	189	61	100.3	1
Genus <i>Prevotella</i>	GGTTCGAGAGGAGGTCCCC	TCCTGCACGGTACTTTGGCTG	100	60	96.1	2
<i>Enterobacteriaceae</i>	CATTGACGTTACCCGAGAGAAGC	CTCTACGAGACTCAAGCTTGG	195	62	94.8	3
<i>Fibrobacter succinogenes</i>	GGTATGGGATGAGCTTGC	GCCTGCCCTGAACATATC	446	60	95.3	4
<i>Clostridium</i> cluster I	ATGCAAGTCGAGCGAKG	TATGGGTATTAATCTYCCCTT	120	60	97.6	5
<i>Clostridium</i> cluster IV	GCACAAGCAGTGGAGT	CTTCCTCCGTTTTGTCAA	130	60	90.8	6
<i>Ruminococcus albus</i>	TGTTAACAGAGGGAAGCAAAAGCA	TGCAGCCTACAATCCGAACATA	75	60	97.7	2
<i>Ruminococcus flavefaciens</i>	GAACGGAGATAATTTGAGTTTACTTAGG	GAACGGAGATAATTTGAGTTTACTTAGG	132	60	91.8	7
<i>Clostridium</i> cluster XIVa	AAATGACGGTACCTGACTAA	CTTTGAGTTTCAITTTCTGGAA	438-441	60	92.2	8
<i>Butyrivibrio fibrisolvens</i>	ACCGCATAAGCCACGGGA	CGGGTCCATCTTGACCGATAAAAT	65	60	96.6	2
<i>Lactobacillus</i> group	AGCAGTAGGGAATCTTCCA	CACCGCTACACATGGAG	341	62	92.8	9
Total methanogens	CCGGAGATGGAACCTGAGAC	CGGTCTTGCCAGCTCTTATTTC	160	60	90.6	10
<i>Methanobrevibacter</i> spp.	TTTCGCCTAAGGATGGTCT	CGATTTCTCACATGCGGAG	171	59	99.7	11
<i>Methanosphaera</i> spp.	TAAGTCTTTGGTGAAAGCTT	GTTACTCACCCGTCGAAGAT	172	61	90.2	11
Total protozoa	GCTTTCGGWTGGTAGTGATTT	CTTGCCCTCYAATCGTWCCT	233	60	90.2	12
<i>Entodinium</i> spp.	GAGTAATACATGCTAAGGC	CCCTCACTACAATCGAGATTTAAGG	317	60	94.7	13
Anaerobic fungi	GAGGAAGTAAAAAGTCGTAACAAAGTTTC	CAAATTCACAAAGGTAGGATGATTT	110-115	60	94.9	7
Urease gene	GCTATCGGTCTCAAACCTTCAYGARGAYTGGGG	GCAATACCATGCCCAATCGCNGCNGGRTTDTATNGT	625	60	79.0	14

¹A_T = annealing temperature; Eff = efficiency.

²References: 1 = Muyzer et al., 1993; 2 = Stevenson and Weimer, 2007; 3 = Bartosch et al., 2004; 4 = Tajima et al., 2001; 5 = Rintillä et al., 2004; 6 = Matsuki et al., 2004; 7 = Denman and McSweeney, 2006; 8 = Matsuki et al., 2002; 9 = Heilig et al., 2002; 10 = Zhou et al., 2009; 11 = Goberna et al., 2010; 12 = Sylvestre et al., 2004; 13 = Skillman et al., 2004; 14 = Baker et al., 2009.