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Effect of Direct-Fed Microbials and Monensin on *In vitro* Fermentation of a High-Forage Diet

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Abstract

An *in vitro* experiment was conducted to determine effects of direct-fed microbials (DFM) on rumen fermentation of a forage-based diet in the presence and absence of monensin (MON). Treatments included DFM (0 and 50,000 cfu; primarily *Lactobacillus acidophilus* and *Enterococcus faecium*) and monensin (0 and 5 ppm) and were applied directly to the appropriate flask. Substrates (80:20 forage: concentrate) were incubated for 30 h. Measurements included serial gas pressure, and terminal CH_4 production, pH, and volatile fatty acid (VFA) and NH_3 concentrations. Interactions between DFM and MON were absent (P>0.10) for gas production and fermentation end products, except for a tendency for DFM to increase total VFA concentration without, but not with MON (interaction between DFM and MON; P=0.07). Addition of DFM did not affect (P>0.10) gas production or fermentative end products except for a tendency (P=0.08) for a slight increase in proportion of isovalerate. >Contrariwise, MON decreased (P<0.001) total gas and CH_4 production. Similarly, MON decreased (P<0.001) total VFA and NH_3 concentrations, and molar proportions of acetate and butyrate and increased (P<0.001) proportions of propionate, valerate, isobutyrate, and isovalerate. Independently, DFM and MON increased (P<0.001) end point pH, resulting in an additive effect when the two treatments were combined. Addition of MON altered fermentation in a predictable fashion and was not influenced by the addition of DFM. In contrast, fermentation characteristics were largely unaffected by DFM with two exceptions: a slight increase in total VFA in the absence of MON and a small increase in pH that appeared to occur through a different mechanism than that for MON.

Keywords: Direct-fed microbial; Monensin; *In vitro* gas production; Methane; VFA

Introduction

Inclusion of DFM in both receiving and finishing diets in beef cattle has been shown to improve intake, as well as rate and efficiency of gain [1-3]. Although the mechanism(s) responsible for these improvements in performance have not been completely elucidated, there is evidence that DFM alter rumen fermentation characteristics (i.e. VFA concentrations, methane production, microbial population) [4-7]. Most commonly, a mixed bacterial culture of lactic acid producing and utilizing, gram positive bacteria are used as DFM in ruminants [4]. Lactic acid producing bacteria such as *Lactobacillus* and *Streptococcus* [8], and lactate utilizing bacteria such as *Megasphaera elsdenii* [9] has been investigated individually as well as in combination to identify their specific beneficial effects on the rumen environment. Recent research from our laboratory has shown that mixed bacterial cultures consisting primarily of *Lactobacillus acidophilus* and *Enterococcus faecium* when used in combination with a high concentrate ration, decreases *in vitro* gas production as well as total VFA concentration [10]. Additionally, *in vivo*, this mixed culture DFM decreased total VFA concentration and increased molar proportions of acetate leading to an increase in pH [11]. However, little is known about this mixed culture DFM (consisting primarily of *Lactobacillus acidophilus* and *Enterococcus faecium*) in combination with a forage-based diet.

Microbial populations are altered by changes in dietary composition leading to higher counts of fibrolytic bacteria with increased forage levels [12]. *Fibrobacter succinogenes* (gram -ve), *Ruminococcus flavefaciens* (gram +ve) and *Ruminococcus albus* (gram +ve) are considered representative fibrolytic bacteria of the rumen [13,14]. These shifts in microbial population have the potential to alter VFA production and subsequently pH levels in the rumen.

Ionophores are a feed-grade antibiotic used in cattle diets to enhance feed efficiency and body weight gain. Ionophores selectively inhibit the metabolism of gram-positive bacteria, which lack a protective outer membrane, and protozoa in the rumen. Monensin is a carboxylic polyether ionophore [15] that has been shown to alter ruminal fermentation by selecting against hydrogen-producing bacteria reducing the substrate for methane production [16]. Moreover, monensin decreases NH_3 production through inhibition of the hyper-ammonia-producing bacteria, a small group of ruminal bacteria that are responsible for the production of most of the ammonia [17]. Additionally, monensin elicits changes in VFA profile, specifically increasing propionic acid production and reducing acetic acid [18-20].

Monensin is widely used in receiving cattle rations as well as in some forage-based production systems [21,22]. The biological benefits of DFM have led to an increase in use. However, little is known about the effects of combined DFM and monensin treatments. Monensin's ability to selectively inhibit gram-positive bacteria in combination with the gram-positive nature of DFM suggests the possibility of a direct interaction between there dietary addition. However, other possibilities for interaction between the two exist, given that both have potential to modify the microbial ecosystem in a variety of ways. The objective of this study was to determine the effect of a mixed bacterial culture, previously used by our lab, consisting primarily of *Lactobacillus acidophilus* and *Enterococcus faecium*, on *in vitro* fermentation and methane production from a forage substrate, with and without the addition of monensin.

Materials and Methods

All procedures were approved by the University of Kentucky Institutional Animal Care and Use Committee.

Donor animals and diet

Four ruminally cannulated steers (396-440 kg) were housed indoors in individual pens (2.4 × 2.6 m) and fed an 80:20 forage: concentrate diet (Table 1) at 1.75 × NEm. The steers were adapted to the experimental diet over a 15 d span prior to sampling of rumen contents and were fed twice daily (8am and 5pm) during the experimental period.

Treatments

All treatments were applied *in vitro* and were arranges as a 2 × 2 factorial. Treatments included 2 levels of DFM (0 and 50,000 cfu) and 2 levels of monensin (0 and 5 ppm). The DFM contained a mixed culture of bacteria consisting of primarily *Lactobacillus acidophilus* and *Enterococcus faecium* and also included *Pediococcus acidilaticii, Lactobacillus brevis,* and *Lactobacillus plantarum* (10-G, Vit-E-Men Co., Norforlk, NE, USA; precise proportions are proprietary). Media DFM dosage (cfu/unit of dry matter) was based on previous *in vivo* studies with the same DFM that have shown enhanced animal performance and alterations in rumen fermentation [23]. Direct-fed Microbial treatments were prepared by dissolving 0.05 g DFM plus carrier or carrier (lactose) into 100 mL distilled water and 1.0 mL of each solution was added to the appropriate vessels. Similarly, monensin treatments were prepared by dissolving 473.2 mg monensin sodium salt (CAS 22373-78-0; MilliporeSigma, St Louis, MO) into 47.32 mL 100% ethanol and 50 µL of the monensin containing solution or ethanol was added to the appropriate vessels [24].

In vitro procedures

Ruminal contents were collected from the ventral rumen of each steer approximately 1 h after morning feeding. Contents from each of the four steers were stored in separate insulated containers for transport into the lab. Prior to processing, the entire contents from each individual animal were combined into a large insulated container. The combined ruminal contents were strained through 4 layers of cheesecloth and the resulting fluid, along with a grab sample of whole contents, were processed using an immersion blender for two minutes under a CO_2 headspace. The blended contents were strained a second time through 4 layers of cheesecloth to form the inoculum source for the experiment. *In vitro* gas production was determined on 4 separate days, with each run consisting of 4 vessels/treatment. One run was determined to be an outlier (detailed in description of statistical analyses), resulting in 12 replications per treatment. Buffer solution, micro- and macro-mineral solutions, and reducing solutions were prepared as described previously [25]. A combination of prepared solutions (1475 mL) and 350 mL of rumen inoculum (media solution) were maintained in a 39°C water bath under a CO_2 environment until added to the 250 mL fermentation vessels. Fermentation vessels were supplied with common substrate (400 mg of donor diet (Table 1); the diet was ground with a Wiley Mill to pass through a 1-mm screen. Each fermentation vessel also received 2 mL of H_2O (to prevent suspension of feed particles outside of liquid solution), 100 mL of media solution and appropriate amounts of each treatment solution (1mL DFM solution or lactose carrier, and 50 µL monensin solution or ethanol). Subsequently, vessels were gassed with CO_2 for 30 seconds and then fitted with remote automatic pressure transducers (Ankom RF Wireless Gas Production System, Ankom Technology, Macedon, NY). Based on preliminary tests runs using the same substrate and conditions (data not shown), vessels were incubated in a water bath at 39°C for 30 hours, to ensure plateau gas pressure was reached, and gas pressure was measured at 5-minute intervals. At the completion of the 30-h fermentation, vessels were placed into an ice bath to cease fermentation and gas samples were drawn into10mL red-topped serum vacutainer tubes for methane analysis. After gas sampling, flasks were opened, pH was immediately determined using a portable pH meter (Acorn pH 6 Meter, Oakton Instruments, Vernon Hills, IL, USA), and samples of the culture broth were collected. A 5 mL aliquot of the sample was added to 15mL Nunc screw-cap centrifuge tubes containing 0.5 mL of metaphosphoric acid (25 g/100 mL) and 0.5 mL of volatile fatty acid (VFA) internal standard (1 g/100 mL 2-ethlybutyrate) and frozen for later VFA analysis. Additionally, a 100 µL sample was combined with 3.9 mL phosphoric acid (25 mM H_3PO_4) and frozen for NH_3 analysis.

Table 1: Ingredients and chemical composition of donor diet and *in vitro* substrate.

Ingredient	%, DM basis
Alfalfa, cube	80.00
Cracked Corn	18.95
Soybean Meal	0.50
Choice White Grease	0.05
Urea	0.16
Limestone	0.28
Trace Mineral-Salt[1]	0.08
Vitamin A,D & E Premix[2]	0.0006
Chemical, DM basis	
CP, %	13.0
ADF, %	26.1
NDF, %	34.75
NFC, %	13.6
Ca, %	1.11
P, %	0.17
NE_m, Mcal/kg[3]	1.03
NE_g, Mcal /kg[3]	0.57

[1]Trace Mineral Premix-Salt, not less than 92% not greater than 96%, Zinc 0.55%, Iron 0.93%, Manganese 0.48%, Copper 0.18%, Iodine 0.01%, Selenium 0.01%, Cobalt 0.01% (2653L, Burkmann Feeds, Danville, KY)

[2]Vitamin Premix-Vitamin A 1,818,182 IU/kg, Vitamin D 363,000 IU/kg, Vitamin E 227 IU/kg

[3]Calculated from nutrient content

Sample analysis

Donor diet and *in vitro* substrate was analyzed for chemical content by a commercial laboratory using wet chemistry methods (Dairy One Forage Laboratory, Ithaca, NY). The concentrations of VFA in the culture broth were determined by gas chromatography (6890 Hewlett-Packard, Avondale, PA), fitted with a Supelco 25326 Nukol fused silica capillary column (15m × 0.53mm × 0.05um film thickness; Sigma/Supelco, Bellefonte, PA). To accomplish the analysis, 0.2 µL of sample was injected at 110°C with a 2:1 split. After a 1-minute hold, the temperature will then be increased at 5°C / minute to 125 ºC for 2 minutes. The inlet and injector were set at 260 °C. Samples prepared as described previously [26]. Ammonia-N concentration was determined using a photometric test with enzymatic assay through Konelab analysis (Model 20XTi, Thermo Fisher Scientific, Waltham, MA) following procedures previously described by Kun and Kearny [27]. Additionally, gas samples were analyzed for methane concentration by gas chromatography (6890 Hewlett-Packard, Avondale, PA), fitted with a Supelco stainless steel 40/60 carboxen 1000 packed column (5ft × 1/8in × 2.1mm). Column head pressure was set at 10psi and oven temperature set point was 125°C [28].

Hay and supplement samples were analyzed for nutrient content by a commercial laboratory (Dairy One Forage Laboratory, Ithaca, NY).

Calculations

Head space volume for each incubation flask (206 ± 4.7 mL) was determined by subtracting additions of media, substrate, and treatments from total volume (water displacement).Cumulative gas pressure readings were converted to gas volumes at standard temperature and pressure using the ideal gas law. Converted gas volumes of individual modules were quantified using the best fit model from the evaluation of ten gas production models evaluated by Pitt et al. [29], in addition to an exponential model without a lag period ($V=VF(1-e^{-kt})$ where V=gas volume at time (t) and VF=gas volume at plateau [30]. All model parameters and curve fit statistics were generated using nonlinear least squares methods in MATLAB (Version R2013a, Mathworks, Natick, MA). The best fit curves were determined as those with the lowest RMSE values. The exponential model without lag, described above, was determined to be the best-fit model for gas production data and was used to calculate rate and total production of gas.

Statistical analysis

Exponential model parameters of *in vitro* gas production (gas volume at plateau and rate of gas production) and fermentation end products (VFA, NH_3 and methane) were analyzed using incubation flask as the experimental unit (n=12) and a model appropriate for a randomized complete block design, with blocks representing separate runs of the *in vitro* procedures conducted on each of 4 separate days. The data were analyzed using the GLM procedure in SAS (SAS Inst. Inc., Cary, NC). The model statement included main effects (DFM and Monensin) and their interactions (DFM × MON), as well as block. Multiple replications of treatments within each run permitted initial analysis to evaluate run × treatment interactions. This preliminary analysis revealed run × treatment interactions (P<0.01) for either DFM and/or MON treatments with all fermentative end products, rate of gas production, and end point pH values which highlighted a single run containing spurious values. This run was removed from subsequent analysis. No other run × treatment interactions were detected (P>0.10) and thus, data were analyzed using only three runs and the interaction term was removed from final statistical analysis for all response variables.

Results

The addition of DFM did not affect (P>0.10) the rate or total gas production (Table 2). In contrast, MON decreased (P<0.01) both total gas production as well as rate of gas production (Table 2).

Monensin decreased (P<0.01) total VFA concentration while DFM tended to increase (P=0.06) total VFA concentration (Table 2). However, there was a tendency (P=0.07) for a DFM × MON interaction for total VFA concentration where DFM increased total VFA concentration in the absence but not in the presence of MON. There were no other DFM × MON interactions (P>0.11) for any of the other variables.

The provision of MON altered the concentration of all VFAs (Table 2). Monensin decreased (P<0.01) acetate and butyrate concentrations, and increased (P<0.01) the concentrations of propionate, isobutyrate, isovalerate and valerate. In contrast to MON, there was no effect of DFM on VFA concentrations except for a tendency to decrease (P=0.08) isovalerate concentration. Monensin decreased (P<0.01) the molar proportions of acetate and butyrate and increased (P<0.01) molar proportions of propionate, valerate, isobutyrate and isovalerate. Similar to the effect of DFM on VFA concentrations, the effect of DFM was absent for molar proportion of VFAs with the exception that DFM slightly decreased (P=0.08) the molar proportion of isovalerate. Additionally, MON decreased (P<0.01) the acetate to propionate ratio (4.33 *vs* 3.15), while DFM had no effect (P>0.10).

Ammonia-N was decreased (P<0.01) by MON (Table 2). In contrast, NH_3-N was not impacted (P=0.57) by DFM. Direct-fed microbial and MON increased (P<0.01) end point pH similarly resulting in an additive effect when the two treatments were combined (Table 2). Monensin decreased (P<0.01) total amount and percent of methane produced (Table 2), while DFM had no effect (P=0.61).

Discussion

In vitro techniques attempt to simulate the rumen environment to allow for characterization of treatment-mediated changes within the rumen. Total gas produced is generally increased as substrate disappearance increases indicating a positive relationship between extent of feedstuff degradation and *in vitro* gas accumulation [31]. In the current experiment, DFM treatment, with a mixed bacterial culture of lactate producing DFM primarily consisting of *Lactobacillus acidophilus* and *Enterococcus faecium,* resulted in no significant changes in total gas production or rate of gas production. Monensin decreased both rate and extent of gas production. Baah et al. [32] observed a linear decrease in total gas production after 12 h of fermentation, relative to control, with provision of increasing levels of lactate producing DFM (i.e. *Lactobacillus casei* and *Lactobacillus* lactis) in a 60:40 forage: concentrate barley-silage-based diet. However, similar to the current study, no differences in total gas production were observed for 6, 24, and 48 h fermentations. While limited data is available on gas production measures with the inclusion of DFM in forage-based diets, more is known about the effect of DFM *in vitro* with concentrate diets. Previous research from our lab using the same inoculum DFM treatment, with high concentrate substrate, resulted in a decrease in total gas production, indicating a decrease in substrate degradation and reduced rumen fermentation [11]. Additionally, Baah et al. [32] reported a linear decrease in total gas production with inclusion of previously mentioned DFM in a 10:90 forage: concentrate barley-grain-based diet. In concert with the current findings, this suggests that DFM, particularly lactate producing bacteria, effects on gas production is dependent, at least in part, on time of incubation and substrate fermented.

Table 2: Impact of direct-fed microbial and monensin on *in vitro* fermentative end products.

	Treatment				P-Value			
	(-) DFM		(+) DFM					
	(-) MON	(+) MON	(-) MON	(+) MON	SEM[1]	MON	DFM	DFM × MON
Gas Production								
Rate, hr^{-1}	0.120	0.107	0.121	0.106	0.001	<0.01	0.93	0.15
Plateau, mL	100.82	82.85	101.53	82.65	0.757	<0.01	0.74	0.55
Methane, %	23.85	18.63	23.67	18.21	0.249	<0.01	0.23	0.63
Methane, mL	23.22	14.47	23.26	14.15	0.274	<0.01	0.61	0.52
Total VFA, mM	71.42	65.81	73.20	65.87	0.47	<0.01	0.06	0.07
Acetate	65.76	61.18	66.02	61.18	0.11	<0.01	0.25	0.26
Propionate	15.28	19.40	15.18	19.50	0.09	<0.01	1.00	0.32
Isobutyrate	1.87	1.92	1.84	1.91	0.02	<0.01	0.35	0.57
Butyrate	10.20	9.74	10.16	9.69	0.06	<0.01	0.43	0.90
Isovalerate	3.55	3.69	3.50	3.68	0.02	<0.01	0.08	0.43
Valerate	3.35	4.06	3.30	4.05	0.02	<0.01	0.18	0.53
Molar Proportion moles/100 moles								
Acetate	65.80	61.20	66.00	61.20	0.11	<0.01	0.25	0.26
Propionate	15.30	19.40	15.20	19.50	0.09	<0.01	1.00	0.32
Isobutyrate	1.90	1.90	1.80	1.90	0.02	<0.01	0.35	0.57
Butyrate	10.20	9.70	10.20	9.70	0.06	<0.01	0.43	0.90
Isovalerate	3.50	3.70	3.50	3.70	0.02	<0.01	0.08	0.43
Valerate	3.30	4.10	3.30	4.00	0.03	<0.01	0.18	0.53
Acetate:Propionate	4.31	3.16	4.35	3.15	0.02	<0.01	0.43	0.11
NH_3,mM	20.90	19.60	21.40	18.70	0.4	<0.01	0.57	0.11
pH	6.49	6.57	6.57	6.67	0.02	<0.01	<0.01	0.61

Dissimilar to DFM, there was a positive relationship between gas production and total VFA concentrations with inclusion of MON treatments, indicating a decrease in dry matter disappearance. Although limited data is available on the effect of monensin on *in vitro* gas production with use of forage-based inoculum, more is known about monensin effects on concentrate diets. Quinn et al. [33] reported average total gas production was 5.9% less than control with monensin treatment on *in vitro* fermentation of steam-flaked corn and cottonseed meal substrate. These results were similar to those reported by Callaway and Martin [34], where *in vitro* culture with added monensin had less total gas production than those not receiving monensin.

Although DFM did not mediate a change in gas production parameters, it did tend to increase total VFA production in the absences of MON without altering VFA proportions. Baah et al. [32] observed a linear increase in total VFA production, as compared to control, with provision of increasing levels of *Lactobacillus casei* and *Lactobacillus lactis* after 6 and 12 h of *in vitro* fermentations; however, a linear decrease was observed at 24 h fermentation. In the same study, linear increase in acetate to propionate ratio was observed with increasing DFM provision after 12, 24 and 48 h of fermentation. It is unknown whether similar results would have been observed in the present study given analyses were only obtained on 30 h data. In contrast, Raeth-Knight et al. [35] observed no difference in total VFA concentrations or molar proportions of individual VFA, *in vivo*, among mixed lactate producing and utilizing DFM treatments (DFM1 *Lactobacillus acidophilus* strain LA747 and *Propionibacterium freudenreichii*; DFM2 *Lactobacillus acidophilus* strains LA747and LA45 and *Propionibacterium freudenreichii*) and control treatments.

In contrast to DFM, monensin decreased total VFA concentration and shifted the molar proportion of acetate and propionate such that there was a decrease in the acetate-propionate ratio. Additionally, monensin decreased butyrate levels and increased isobutyrate, valerate and isovalerate levels. Treatment effects were absent for total VFA concentrations in previous research with inclusion of varying levels of monensin in forage based diets [36-38]. Similar to the findings of the present study, Richardson et al. [18] and Ramanzin et al. [37] observed decreased proportions of acetic and butyric acids with concurrent increased proportion of propionic acid with inclusion of monensin *in vitro* and *in vivo* (respectively).

Both DFM and MON are commonly incorporated into cattle diets. However, there is a paucity of information in the literature concerning the potential interaction between DFM and MON. In addition to the main effect of both additives there was a tendency for DFM × MON interaction on total VFA concentration, while no other interactions were observed. DFM tended to increase total VFA concentration in the absence but not in the presence of MON. The ability of DFM to alter total VFA concentrations in the absence but the not presence of MON, suggests that monensin may be impacting the ruminal effects of the DFM. The basic mode of action of an ionophore is to disrupt the movement of ions (Na^+, Ca^{2+}, K^+, H^+) across biological membranes [39]. Ionophores selectively inhibit the metabolism of gram-positive bacteria, which lack a protective outer membrane, and protozoa in the rumen. Monensin's ability to inhibit lactate-producing rumen bacteria,

S. bovis and *Lactobacillus* species, was observed by Dennis et al. [40] in sensitivity and growth rate trials. Similar results were reported in which monensin significantly decreased the *lactobacilli* and *enterococci* counts in *in vitro* incubations of the crop contents of chickens not previously exposed to monensin treatments [41]. This research suggests that monensin may have an adverse effect on the DFM treatments in the present study given the DFM primarily consist of *Lactobacillus acidophilus* and *Enterococcus faecium.* Additionally, Van Nevel et al. [42] reported efficiency for both total and net microbial growth were significantly depressed by addition of monensin in an *in vitro* study as determined by measuring the incorporation of ^{32}P-labeled phosphate in microbial material, which is in agreement with other data obtained *in vitro* as well as *in vivo* [19]. This would suggest that monensin could either directly inhibit the DFM bacteria and/or other bacteria that may be positively influenced by the DFM but inhibited by monensin.

In the present study, monensin decreased total VFA concentration and increased end point pH levels of culture broth. These observations are inconsistent with previous *in vivo* studies in cattle consuming high forage diets which have shown the inclusion of monensin to have no effect on total VFA concentration or rumen pH levels [38,43]. The reason for this discrepancy is unclear. In contrast, inclusion of DFM treatment resulted in increased pH of the culture broth combined with increased total VFA concentrations, particularly in the absence of monensin. Nonetheless, it has been well demonstrated across a variety of diets that there is an inverse relationship between rumen pH and total VFA concentrations [44,45]. Other research reported no difference in rumen pH with inclusion of various strains and combinations of *Lactobacillus acidophilus and Propionibacterium* in forage-based diets [35,46]. Given the scope of this experiment it is difficult to ascribe a mechanism to account for the currently observed increase in both pH and total VFA concentrations. Inclusion of the current DFM has resulted in increased levels of total VFA in combination with decreased pH, *in vitro*, with high concentrate substrate [11]. This is an indication that DFM-modulated changes could be dependent on diet.

Monensin reduces NH_3-N concentration through inhibition of the hyper-ammonia producing bacteria; a small group of ruminal bacteria that are responsible for the production of most of the NH_3 [17]. In the present study monensin decreased levels of NH_3 when used *in vitro* with high forage substrate. Similar results were observed *in vitro* using a timothy hay substrate with the inclusion of monensin, where monensin caused a significant decrease in NH_3 accumulation [47]. Additionally, monensin appeared to decrease NH_3 concentrations levels *in vivo* with a 90% orchardgrass diet although the differences among treatments were not significant [43]. Ammonia-N did not differ with provision of DFM, which is in agreement with previous work that has found no differences in NH_3 concentrations or microbial N with DFM provision *in vitro* and *in vivo* [32,35].

Monensin decreased methane concentrations with inclusion *in vitro* with a high forage substrate. Similar results have been observed *in vitro* using a timothy hay substrate with the inclusion of monensin, where monensin caused a significant decrease in methane production [47]. Additionally, monensin decreased methane production *in vivo* when included in forage-based diets consisting of corn silage/haylage and barley silage [48,49]. Benefits of feeding monensin include a shift in the acetate to-propionate ratio toward more propionate and an associated decrease in methanogenesis [50]. Consistent with these findings, monensin decreased methane and increased propionate in the present study.

There was no significant effect of DFM on methane production. Increased levels of acetate are indicative of increased methane production (as previously mentioned); consistent with our results neither end product was affected by DFM provision. Limited data is available with direct measure of methane as affected by inclusion of DFM. However, it has been suggested that some DFM may redirect H_2 reducing its availability for use in methane production [51]. In terms of DFM containing yeast, there have been inconsistent results from studies both *in vitro* and *in vivo,* however, live yeast have shown beneficial effects on the growth and H_2-utilization of acetogenic bacteria *in vitro* [52]. Acetogenic bacteria reduce carbon dioxide to acetate and in this reduction they are competing with methanogens for hydrogen subsequently reducing CH_4 production [53]. An additional strategy to mitigate CH_4 in ruminants is to increase competition for hydrogen by producing more propionate in the rumen [49,54]. A recent study conducted by Alazzeh et al. [53] provides evidence those specific strains of *Propionibacteria,* which produce propionate as an end product of fermentation, could help mitigate methane production *in vitro* with forage diets.

Conclusion

The addition of monensin inhibited fermentation as indicated by reductions in both gas production and total VFA concentration. Monensin also altered VFA proportions and reduced methane production as has been previously reported. These actions were not influenced by the addition of DFM. In contrast, DFM had little influence on fermentation characteristics; exceptions being a slight increase in total VFA in the absence of MON and a small increase in pH that appeared to occur through a mechanism that differs from that for MON. Both DFM and MON increased pH, resulting in an additive effect when the two treatments were combined.

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b. Mention of trade name, proprietary product, or specified equipment does not constitute a guarantee or warranty by the University of Kentucky and does not imply approval to the exclusion of other products that may be available.

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