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ORIGINAL ARTICLE

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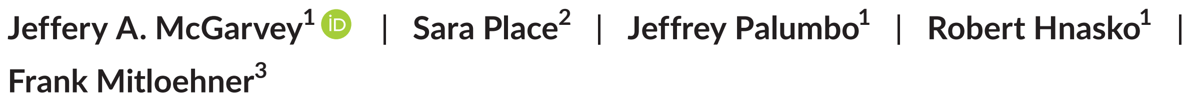
Dosage-dependent effects of monensin on the rumen microbiota of lactating dairy cattle



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Abstract

We examined the dose-dependent effects of feeding lactating dairy cows a standard diet supplemented with monensin at 175, 368, or 518 mg cow^{-1} day^{-1}on the rumen microbiota. For each dosage, 3 animals were randomly assigned into groups and fed the same basal total mixed ration diet supplemented with monensin, at the respective dose. After 20 days, rumen samples were taken and the effect on the microbiota was examined by 16S rRNA gene sequence analysis and qPCR. At the lowest dose no significant change in 16S rRNA gene sequences associated with any bacterial phyla was observed; however, at the medium and high dosages, we observed significant reductions in sequences associated with gram-positive bacteria and significant increases in those associated with gram-negative bacteria that were dosage dependent. All dosages reduced the levels of sequences associated with methanogenic archaea in the rumen, with the medium dosage showing the largest decline. No significant difference was observed for the 18S rRNA gene sequences associated with protozoa in any of the libraries. Our results indicate that with this diet the medium dosage of monensin was most efficacious for the reduction in methanogenic archaea in the rumen of lactating dairy.

KEYWORDS

16S rRNA, dairy cattle, microbiota, monensin, Rumen

1 | INTRODUCTION

Monensin is a carboxylic polyether ionophore antibiotic (Haney & Hoehn, 1968) produced by *Streptomyces cinnamonensis* that was originally developed as a coccidiostat for chickens (Russell & Strobel, 1989). However, in the 1970s studies showed that beef cattle fed monensin had greater feed efficiency due to alterations in ruminal fermentation (Dinius, Simpson, & Marsh, 1976; Richardson, Raun, Potter, Cooley, & Rathmacher, 1976). Specifically, cattle fed monensin produced greater amounts of propionate that can be converted to glucose in the liver *via* gluconeogenesis (Huntington, 1990), and less methane (Thornton & Owens, 1981), which is not only a potent greenhouse gas but represents a gross energy loss of 2%–12% for the animal (Johnson & Johnson, 1995). Monensin treatment also reduces protein degradation in the rumen (Whetstone, Davis, & Bryant, 1981), allowing more feed protein to reach the abomasum (Falkner, Klopfenstein, Trotter, & Britton, 1985), where it is converted to amino acids that are absorbed in the intestine. In addition, monensin reduces the incidence of digestive disorders such as bloat (Bartley et al., 1983), acidosis (Dennis & Nagaraja, 1981), and ketosis (Sauer, Kramer, & Cantwell, 1989) and in dairy cows increases yields of milk and milk protein (McGuffey, Richardson, & Wilkinson, 2001).

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Monensin's ability to effect these changes is due to its ability to alter the microbial populations within the rumen. Monensin achieves this by inserting itself into susceptible cell membranes where it acts as a metal/proton antiporter (Pressman, 1976), eliminating the H^+, Na^+, and K^+ ion gradients across the cell membrane, resulting in the collapse of the proton motive force and eventually cell death (Russell, 1987; Russell & Strobel, 1989). Monensin is most effective against gram-positive bacteria, because they lack the protective outer membrane of gram-negative cells (Russell & Strobel, 1988). Thus, animals fed monensin have higher levels of gram-negative bacteria that are more likely to produce propionate; and reduced levels of gram-positive bacteria, that are more likely to degrade dietary protein (Russell & Strobel, 1988) and supply methanogens with hydrogen and formate for methanogenesis (Russell & Strobel, 1989). However, studies have shown that monensin supplementation does not always produce these effects (Hamilton, DePeters, McGarvey, Lathrop, & Mitloehner, 2010; Hook, Northwood, Wright, & McBride, 2009; McGarvey, Hamilton, DePeters, & Mitloehner, 2010; Odongo et al., 2007). Several studies have suggested that monensin's ability to alter the microbial populations within the rumen is dependent on both animal diet (Grainger, Williams, Eckard, & Hannah, 2010; Guan, Wittenberg, Ominski, & Krause, 2006) and monensin dosage (Duffield, Merrill, & Bagg, 2012; Ellis et al., 2012). However, there have not been any studies to date that have examined the dose-dependent effects of monensin on the rumen microbiota of lactating dairy cows. We hypothesized that we could identify a dosage of monensin that is effective at altering the bacterial population structure of the rumen and thus reduce the levels of methanogenic archaea by feeding lactating cows a standardized diet supplemented with increasing levels of monensin and measuring the changes in the rumen microbiota. In this study, we fed lactating dairy cattle a standardized diet supplemented with three different dosages of monensin and monitored the changes in their rumen microbiota *via* 16S gene sequence analysis and qPCR.

TABLE 1 Composition of diet

Ingredient	% Total
Corn silage	36
Alfalfa hay	18
Whole cottonseed	9
Almond hulls	4
Grain mix[a]	33
Composition	**(g kg^{-1})**
Neutral detergent fiber	376
Acid detergent fiber	267
Starch	183
Crude protein	156
Ash	74
Sugar	56
Lignin	53
Crude fat	52
K	16
Non fiber carbohydrate	9
Ca	4
Mg	4
S	3
Na	3
Fe (mg kg^{-1})	332
Mn (mg kg^{-1})	87
Zn (mg kg^{-1})	76
Cu (mg kg^{-1})	22

[a]Grain Mix: 22% ground corn;18.5% ground wheat; 18.6% soybean hulls; 13.3% corn germ; 8.6% canola meal; 6.1% feather meal; 3.1% megalac; 2% CaCO3; 2% blood meal; 1.6% Na3H(CO3)2; 1.6% NaCl; 1.4% urea; 0.6% MgO; 0.3% PO4; and 0.6% vitamin & mineral premix (Cargill, Minneapolis, MN).

2 | MATERIALS AND METHODS

2.1 | Animals used in the study

Twelve multiparous, lactating Holstein dairy cows from the University of California, Davis dairy herd were used in this study. The animals were housed in a facility approved by the Association for Assessment and Accreditation of Laboratory Animal Care and all protocols were approved by the UC Davis Institutional Animal Care and Use Committee. The 12 animals were randomly assigned into four groups (three cows per group) and were fed the same basal total mixed ration diet, twice daily, that was top-dressed with monensin at each respective dose: Control (0.0 mg cow^{-1} day^{-1}), Low (175 mg cow^{-1} day^{-1}), Medium (368 mg cow^{-1} day^{-1}), or High (518 mg cow^{-1} day^{-1}) for 20 days. The nutritional composition of the diet (Table 1) was analyzed by Cumberland Valley Analytical, Inc. (Hagerstown, MD, USA), and the concentration of monensin was determined by Covance Laboratories (Greenfield, IN).

2.2 | Rumen sampling

Rumen samples were collected from each cow before and after 20 days of monensin treatment to ensure the monensin treatment had enough time to take effect. Rumen fluid was collected from each animal *via* an oral stomach tube as described by Lodge-Ivey, Browne-Silva, and Horvath (2009). Briefly, approximately 2.5 hr after the feeding, cows were placed in a chute and a steel bovine mouth speculum was placed in the animal's mouth. A plastic stomach tube (0.6 cm I.D. and 3 m length) was inserted through the speculum into the rumo-reticulum and approximately 250 ml of fluid was collected from each animal and transferred into 50 ml tubes (Becton Dickenson, Franklin Lakes, NJ, USA) that were immediately sealed, placed on ice and transported to the laboratory for analysis. Precautions were taken to prevent saliva contamination; however, it is possible that some contamination occurred.

2.3 | 16S rRNA gene library construction

DNA was extracted from 15 ml of rumen fluid as described by Yu and Morrison (2004). PCR amplification of 16S rRNA genes was carried out using the primers 27f (5′ AGAGTTTGATCCTGGCTCAG 3′) and 1392r (5′ GACGGGCGGTGTGTAC 3′) (Lane, 1991). PCR was performed as recommended by Polz and Cavanaugh (1998) to reduce bias in amplification. Briefly, 50 µl reactions contained 25 µl High Fidelity PCR Master Mix (Roche, Nutley, NJ, USA), 50 ng DNA and 1 µmole L^{-1} of each primer. PCR was performed in a Tetrad Thermocycler (Bio-Rad, Hercules, CA, USA) under the following conditions: one cycle of 95°C for 5 min, 20 cycles of 94°C for 30 s, 55°C for 30 s, 72°C for 1.5 min, and one cycle of 10 min at 72°C. The PCR products were visually examined *via* agarose gel electrophoresis to ensure a single 13.5 Kbp band was produced, purified using the Zymo DNA Clean and Concentrator Kit (Zymo Research, Orange, CA, USA), cloned using the TOPO TA Cloning Kit (Invitrogen, Carlsbad, CA, USA) and transformed into *E. coli* TOP10 competent cells (Invitrogen). Clones were grown on LB agar (Fisher Scientific, Fair Lawn, NJ, USA) plates containing kanamycin (Km) (50 µg ml^{-1}) at 37°C for 18 hr. Colonies were transferred to 96-well plates with LB Km broth (Fisher Scientific). For each treatment, three animals were sampled for rumen fluid that was extracted for DNA. For each DNA sample, two 96-well plates of sequences were analyzed, for a total of 576 sequences for each treatment group, and 2,304 sequences in total.

2.4 | DNA template preparation and sequencing

DNA templates were prepared from 0.2 µl overnight cultures using the TempliPhi HT Amplification Kit (GE Healthcare, Piscataway, NJ, USA). Sequencing reactions were performed using the primer 1392r and the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA). Sequencing reactions were purified using the BigDye XTerminator Purification Kit (Applied Biosystems); electrophoresis and readout were performed using an Applied Biosystems 3730XL Genetic Analyzer (Applied Biosystems).

2.5 | DNA sequence and statistical analysis

DNA sequences were edited manually to correct falsely called bases and trimmed at both the 5′ and 3′ ends using the Seqman software (DNASTAR Inc., Madison, WI, USA) and analyzed for chimeras using UCHIME (Edgar, Hass, Clemente, Quince, & Knight, 2011). Sequences with reads of 600 bp covering hypervariable regions V5-V8 were grouped into operational taxonomic units (OTU) (>97% sequence identity) using the FastGroup (Sequritan & Rohwer, 2001). Each OTU was assigned to a phylum using the Classifier software (Wang, Garrity, Tiedje, & Cole, 2007), available at the Ribosomal Database Project II (http://rdp.cme.msu.edu/index.jsp). Once classified, pair-wise comparisons of the OTU were performed using the Ribosomal Database Project II Library Compare software (Wang et al., 2007). Sequences of all OTU were deposited in GenBank under submission SUB2986750. Simpson entropy, Chao1 estimates, and evenness were calculated using Estimate S (Colwell, 2006).

2.6 | Quantitative PCR

Quantitative polymerase chain reaction (qPCR) for the methanogenic archaea DNA in rumen fluid was performed as described by Ohene-Adjei et al. (2008) and for protozoa, it was as described by Sylvester, Karnati, Yu, Morrison, and Firkins (2004). For both experiments, standard curves of template DNA (methanogen or protozoan) were made to contain 1.0×10^8–1×10^5 copies per µl in 10-fold serial dilutions. All qPCR was performed in triplicate using an Applied Biosystems 7500 Real-Time PCR System (Life Technologies, Carlsbad, CA, USA). Significance of the qPCR was determined by the Student's t test with $p < 0.05$. Primers used for archaea were MB1174F GAGGAAGGAGTGGACGACG GTA and Arch1406R ACGGGCGGTGTGTGCAAG (Ohene-Adjei et al., 2008); primers used for protozoa were 316f GCTTTCGWTGGTAGTGTATT and 539r CTTGCCCTCYAATCGTWCT (Sylvester et al., 2004). Efficiency of PCR was calculated by the equation $E = -1 + 10^{(-1/\text{slope})}$ and ranged between 89% and 108%.

3 | RESULTS AND DISCUSSION

3.1 | 16S rRNA analysis

To determine the effects of monensin on the bacterial populations within the rumen we constructed 16S rRNA gene libraries from DNA extracted from rumen fluid of cows fed an identical diet (Table 1) supplemented with either no, low (175 mg cow^{-1} day^{-1}), medium (368 mg cow^{-1} day^{-1}), or high (518 mg cow^{-1} day^{-1}) doses of monensin for 20 days (Table 2). The library derived from the rumen fluid of the cows fed the control diet (i.e., no monensin) contained sequences representative of 10 phyla (Table 2). The majority of these sequences were associated with the phyla *Bacteroidetes* (58.9%), *Firmicutes* (31.4%), and *Proteobacteria* (3.6%). These results are similar to previous studies that examined 16S rRNA genes libraries derived from lactating dairy cows (Kasparovska et al., 2016; Pitta et al., 2016). The library derived from the cows fed the low dose of monensin had no significant change ($p > 0.05$) in the percentage of sequences associated with any of the phyla. The library derived from the cows fed the medium dose had a significant increase in sequences associated with the phylum *Bacteroidetes* and a significant decrease in those associated with the *Firmicutes* ($p < 0.05$). The library derived from the cows fed the high dose of monensin contained a significant increase in sequences associated with the phyla *Bacteroidetes* and *Proteobacteria* and significant decrease in those associated with the phylum *Firmicutes*.

These dose-dependent changes in the bacterial populations are consistent with in vitro studies that showed the antibacterial activity of monensin is most effective against gram-positive bacteria (Chow & Russell, 1990; Russell, 1987). While monensin is known to attach equally well to both gram-positive and gram-negative

TABLE 2 Percentage of 16S rRNA gene sequences associated with bacterial phyla

	Monensin dosage			
Phylum	Control	Low	Medium	High
Actinobacteria	0.0	0.2	0.2	0.2
Bacteroidetes	58.9	62.8	65.8*	65.9*
Ca. Saccharibacteria	0.2	0.0	0.4	0.6
Chloroflexi	0.2	0.0	0.2	0.0
Elusimicrobia	0.0	0.0	0.0	0.2
Firmicutes	31.4	26.8	22.5*	19.0*
Fibrobacteres	1.3	1.8	0.6	0.4
Lentisphaerae	0.2	0.0	0.0	0.0
Planctomycetes	0.0	0.2	0.0	0.0
Proteobacteria	3.6	4.2	4.7	7.1*
Spirochaetes	1.7	0.4	1.3	1.8
Synergistetes	0.0	0.0	0.2	0.2
Tenericutes	0.4	0.6	0.4	0.2
TM7	0.4	0.0	0.4	0.6
Verrucomicrobia	0.0	0.0	0.2	0.6
Unclassified	2.1	3.2	3.4	3.8

*Significant difference from control library ($p < 0.05$)

bacteria (Chow, Kessel, & Russell, 1994), its effectiveness against the gram-positive bacteria is believed to be due to their lack of an outer membrane (Russell & Strobel, 1988). However this may be an oversimplification, as some gram-positive bacteria have been shown to become adapted to monensin in vitro (Simjee, Heffron, Pridmore, & Shryock, 2012; Weimer, Stevenson, Mertens, & Hall, 2011) as-well-as in vivo (Weimer, Stevenson, Mertens, & Thomas, 2008). The adaptation of gram-positive bacteria to monensin exposure has been associated with alterations in protein production, cell wall structure (Simjee et al., 2012) and the production of extracellular polysaccharides (Rychlik & Russell, 2002; Weimer et al., 2008). However, there are no resistance genes associated with this phenotype, and it is rapidly lost when selective pressure is removed (Simjee et al., 2012), suggesting it is epigenetic.

TABLE 3 Diversity statistics

Library (dosage)	No. of clones	Richness (No. OTU)	Chao1 estimate	Simpson Index (1/D)	Evenness Index (*E*)
Control	518	311	1,008	180	0.942
Low	492	302	755	268	0.957
Medium	500	313	761	252	0.956
High	502	311	755	167	0.940

Monensin treatment also resulted in changes in the bacterial diversity within the rumen (Table 3). Feeding all doses of monensin reduced the number of operational taxonomic units (OTU), as estimated by Chao1 analysis, by approximately 25%. The Simpson indices, which incorporate species richness (or in this case OTU richness) and evenness, were also affected by the addition of monensin to the diet. At the low dose an increase in these indices was observed, and as the doses increased these indices decreased in a dose-dependent manner. This effect on the microbial diversity in the gut of animals fed antibiotics has been observed previously (Looft et al., 2012; Suchodolski et al., 2009).

3.2 | Methanogenic archaea qPCR

Monensin has been shown to have little or no direct effect against methanogenic archaea (Russell & Houlihan, 2003); however, the decreases in gram-positive bacteria within the rumen has been shown to reduce the concentration of methanogenic substrates (i.e., hydrogen and formate) needed by these microorganisms (Haney & Hoehn, 1968; Russell & Strobel, 1989). To determine the effect of monensin on the number of methanogens in the rumen, we performed qPCR on DNA extracted from the rumen fluid (Table 4). In the control group, no significant change was observed in methanogen sequences present after 20 days. However, significant decreases were observed for all levels of monensin treatment ($p < 0.01$). At the low and medium dose, we observed a 3.9- and 7.5-fold decrease in these sequences, respectively. Interestingly, the high dose only decreased these sequences by ~twofold. These data are consistent with those of Hook et al. (2009), who observed a decrease in the number of methanogen sequences in rumen fluid after 20 days of monensin treatment. However, after 90 days of treatment, the methanogens recovered and no significant effect was observed over their 180-day experiment. Likewise, Guan et al. (2006) reported a significant decrease in methane production after short-term monensin treatment; however, normal methane production resumed after 4 weeks, leading the authors to speculate that the rumen bacteria had adapted to monensin.

TABLE 4 Methanogen 16S rRNA gene copies ng^{-1} DNA before and after monensin treatment

Animal group	Before (*SD*)	After (*SD*)	*p*-Value
Control	3.71×10^6 (4.37×10^5)	3.02×10^6 (2.43×10^6)	0.420
Low	5.13×10^6 (2.06×10^6)	1.32×10^6 (8.64×10^5)	0.0001
Medium	4.13×10^6 (1.19×10^6)	5.49×10^5 (1.90×10^5)	0.0001
High	5.42×10^6 (2.35×10^6)	2.76×10^6 (1.01×10^6)	0.0075

Note. *SD*: standard deviation.

TABLE 5 Protozoan 18S rRNA gene copies ng^{-1} DNA before and after monensin treatment

Animal group	Before (*SD*)	After (*SD*)	*p*-Value
Control	1.24×10^7 (8.32×10^6)	1.71×10^7 (2.69×10^6)	0.75
Low	6.68×10^6 (2.46×10^6)	2.91×10^6 (3.68×10^6)	0.12
Medium	5.68×10^6 (2.83×10^6)	9.03×10^6 (9.81×10^6)	0.54
High	1.38×10^7 (6.51×10^6)	8.34×10^6 (1.88×10^6)	0.33

Note. *SD*: standard deviation.

3.3 | Protozoa qPCR

It is estimated that up to 25% of rumen methanogens are associated with protozoa (Newbold, Lassalas, & Jouany, 1995) that supply them with H_2 and CO_2 via their hydrogenosomes (Embley, Giezen, Horner, Dyal, & Foster, 2003) that they convert to methane, water, and energy (Wolin, 1974). To determine if the decreases in methanogens were related to the anti-protozoan activity of monensin, we performed qPCR to quantify the number of protozoan 18S rRNA genes extracted from rumen fluid before and after monensin treatment. Our results showed that monensin had no significant effect on the number of protozoan 18S rRNA genes in the rumen fluid at any dosage tested (Table 5).

Overall, the high dosage of monensin produced the greatest increase in the gram-negative phyla *Bacteroidetes* and *Proteobacteria* and the greatest decrease in the gram-negative phylum *Firmicutes*. However, the middle dosage also produced significant alterations in the phyla *Bacteroidetes* and *Firmicutes* and was more effective at reducing the methanogenic archaea than the high dosage. From these data, we conclude that for lactating dairy cows fed this diet the middle dosage was the most efficacious. Future studies are needed to examine the effects of these dosages on milk production and animal health to determine the economic return of using these dosages with this diet and to examine the possibility of bacterial adaptation to monensin over time.

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CONFLICT OF INTEREST

No conflict of interest declared.

AUTHORS CONTRIBUTION

JM, SP, JP, and RN carried out bench work, data analysis and were involved in the manuscript preparation. SP and FM designed the study and performed all work related to sampling and care of the animals. All authors have read and approved the final manuscript.

ETHICS STATEMENT

The animals were housed in a facility approved by the Association for Assessment and Accreditation of Laboratory Animal Care and all protocols were approved by the UC Davis Institutional Animal Care and Use Committee.

DATA ACCESSIBILITY

All sequences were deposited in GenBank and are available under submission SUB4783994 accession numbers MK161521-MK163307.

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