Evaluation of the microbial population in ruminal fluid using real time PCR in steers treated with virginiamycin

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ABSTRACT: The objective of the present study was to investigate the effects of virginiamycin (VM) supplementation on ruminal fermentation and microbial populations in steers. Four ruminally cannulated Chinese Luxi steers (BW 559.4 ± 30.1 kg) were used in a crossover design experiment with an experimental period of 28 days. The forage to concentrate ratio of the basal diet was 35:65 on dry matter basis. The experiment consisted of control treatment and treatment with control diet plus VM at a dose of 30 mg/kg concentrate (DM basis). Rumen fluid was collected at 07:30 prefeeding, at 11:30 and 17:30 postfeeding on day 27 and 28. A part of the pooled sample from rumen fluid was transferred to anaerobic culture by a roll-tube technique and analysed for species-specific real-time PCR quantification. The remaining pooled rumen fluid sample was analyzed for pH, VFA, ammonia N and L-lactic acid. The results showed that VM increased the ruminal pH (6.70 vs. 6.63; $P < 0.05$), but it decreased ammonia nitrogen (4.94 vs. 6.19 mg/100 ml; $P < 0.01$) and mean counts of amylolytic bacteria and proteolytic bacteria ($P < 0.01$) as compared to the control. The additive VM did not affect the L-lactic acid concentration (1.39 vs. 1.26 mmol/l) in rumen fluid. Compared to the control, the steers receiving VM have altered a trend of quantification of *Selenomonas ruminantium*, *Anaerovibrio lipolytica*, *Ruminococcus albus* and *Streptococcus bovis* in rumen fluid ($0.05<p<0.1$) as compared to the control. However, VM had no significant effect on *Lactobacillus* spp. ($P = 0.41$), *Butyrivibrio fibrisolvens* ($P = 0.35$), on the genus *Ruminococcus* ($P = 0.25$), *Ruminococcus flavefaciens* ($P = 0.52$), *Prevotella ruminicola* ($P = 0.54$), on the genus *Prevotella* ($P = 0.67$) and *Megasphaera elsdenii* ($P = 0.97$). In this study, we found that VM had selective effects on ruminal bacteria and influenced ruminal fermentation by changing a part of the specific ruminal bacteria populations.

Keywords: virginiamycin; roll-tube technique; ruminal fermentation parameters; real-time PCR

Virginiamycin (VM) is an antimicrobial feed additive approved for use in cattle to improve performance. The inclusion of VM in diets reduces the risk of lactic acidosis in feedlot cattle (Rowe et al., 1994; Rogers et al., 1995), stabilizes ruminal pH and increases digestibility and energy utilization of grains (Godfrey and Pethick, 1992). VM seems to control the growth of ruminal lactic acid-producing bacteria, therefore, it has the potential to moderate ruminal fermentation in situations that could lead to rapid production of lactic acid. It is believed to alter ruminal fermentation primarily by changing ruminal microbial populations. VM is an antibiotic active against Gram-positive bacteria, including

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Streptococcus bovis and *Lactobacillus* spp., which produce lactic acid. The Gram-positive bacteria antimicrobial activity and subsequent alterations in ruminal fermentation products are similar to those of monensin (Hedde et al., 1982; Nagaraja et al., 1997). *In vitro* studies have shown that VM is a potent inhibitor of lactic acid production because of the inhibition of lactic acid-producing ruminal bacteria (Nagaraja and Taylor, 1987; Nagaraja et al., 1987). However, a few literary sources are available on the effects of VM on specific ruminal microbes and alteration in ruminal fermentation due to its influence on ruminal microbial populations.

The objective of this study was to determine the effects of VM on the populations of specific ruminal bacteria and to determine if any population shift was consistent with the envisaged mode of VM action. By using the real-time polymerase chain reaction (RT-PCR) with taxon-specific primers and an absolute quantification procedure, the population of individual taxa was determined with accuracy (Stevenson and Weimer, 2007).

MATERIAL AND METHODS

Animals and treatments

Four rumen cannulated steers (body weight 559.4 ± 30.1 kg) were used in a crossover design with 28 days of each treatment period. Steers were randomly assigned to the control (no VM; forage to concentrate ratio 35:65 on dry matter basis) and to a treatment group in which VM premix-Stafac®500 (60 mg/kg concentrate, dry matter basis; Stafac®500 composition: 50% VM; Phibro Corporation Ltd.) was added to the diet. The diet was formulated as per National Research Council standards for maintenance requirement of steers (Table 1). The diet was fed twice daily in equal portions at 08:00 and 20:00 hours. The daily feeding was fixed at 7.5 kg/cow and included Chinese wildrye addition (2.5 kg/cow/day). Steers were housed in individual stalls with shelter. At the beginning of the treatment, steers were gradually adapted to the experimental diets over a 14-day period. The VM was mixed evenly with the concentrate before feeding. Animal care and procedures were approved and conducted under established standards of the Institute of Animal Science, Chinese Academy of Agricultural Science.

Sampling and analysis

Ruminal fluid samples for VFA, ammonia N and pH were collected through the cannula in the rumen every 2 hours over a 12-hour period on day 27 of each experimental treatment before morning feeding. Whole ruminal contents were collected at 07:30 prefeeding and at 11:30 and 17:30 hours postfeeding on day 28 of experimental treatment from the anterior, dorsal and mid-ventral regions of the rumen by hand and squeezed through four layers of sterile cheesecloth. The first 100 ml of strained ruminal fluid was discarded. The residual rumen fluid

Table 1. Ingredient and chemical composition of experiment basal diets

Ingredients (g/100 g DM)		Chemical compositions	
Chinese wildrye	35.00	$N_E$mf (Mcal/kg)	0.96
Maize	45.28	CP (%DM)	10.2
Wheat bran	11.05	Ca (% DM)	0.47
Soybean meal	4.12	P (% DM)	0.23
Cottonseed meal	2.82	ADF (%DM)	25.52
NaCl	0.65	NDF (%DM)	38.77
Calcium carbonate	0.43		
Premix[a]	0.65		

[a]each kilogram contains VA ≥ 1 000 000 IU; VD3 ≥ 65 000 IU; VE ≥ 5 000 mg; Fe ≥ 2 000 mg; Cu ≥ 1 750 mg; Zn ≥ 5 500 mg; Mn ≥ 2 550 mg; Se ≥ 75 mg; I ≥ 70 mg and Co ≥ 40mg

was immediately used to measure pH (370 model pH meter, Jenway, UK). Ruminal fluid samples were used to analyze ammonia N (Broderick and Kang, 1980; Jalč and Čertík, 2005) and VFA concentration (Vanzants and Cochran, 1994; Jalč et al., 2009). A French Biosentec kit (Cat. No. 022, Arrow Scientific) was used to analyze L-lactate (Bramley et al., 2008). The total and individual bacterial counts (cellulolytic, proteolytic, amylolytic bacteria, and total viable bacteria) were assessed using the roll-tube technique (Hungate, 1969), and protozoa were counted under a microscope (Boyne et al., 1957; Dehority, 1984; Váradyová et al., 2007) at an interval of 0, 4 and 8 h after morning feeding. The rumen content samples for DNA extraction were obtained via the fistula at 11:30 and 17:30 hours postfeeding on day 28 of experimental treatment. The whole ruminal contents from the anterior, dorsal, and mid-ventral regions of the rumen by hand were squeezed through four layers of sterile cheesecloth. The strained ruminal fluid was centrifuged at 15 000 × g for 15 min at room temperature. The supernatant was discarded and the sediment was immediately dissolved in sterile normal saline and stored at –80°C. Samples for bacterial groups and DNA extraction were taken on the same day as for VFA analysis.

DNA extraction

DNA was extracted from ruminal fluid by a bead method according to Yu and Morrison (2004). Briefly, 0.5 ml ruminal fluid was mixed with 1 ml lysis buffer (500 ml Tris-HCl, pH 8.0, 50mM EDTA and 4% sodium dodecyl sulphate /SDS/) and 0.4 g of sterile zirconia beads (0.3 g of 0.1 mm and 0.1 g of 0.5 mm). After beating in a Mini-Beadbeater (Retsch, Germany) for 3 min at maximum speed, the sample was incubated at 70°C for 15 min. The most of the impurities and SDS were removed by precipitation with 10 M ammonium acetate and then the nucleic acids were recovered by precipitation with isopropanol. Genomic DNA was then subjected to sequential digestions with RNase and proteinase K and further purified with a DNA clean-up kit (Takara, Japan). Concentrations of DNA were measured with NanoDrop 1000 (Thermo Scientific, German). On average, the DNA used for these experiments possessed an A260/A280 ratio of 1.8.

PCR amplification, cloning and sequencing

Equal amounts of DNA from each animal were combined into a single sample for each treatment and then amplified using the following procedures. A pair of universal primers was used for the PCR reaction to amplify almost the full length 16S rDNA sequences: 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3') (Gurtler and Stanisich, 1996). PCR amplification was performed as follows: 95°C for 4 min, followed by 15 cycles consisting of 94°C for 45 s, 56°C for 50 s and 72°C for 2 min, and a final extension period of 72°C for

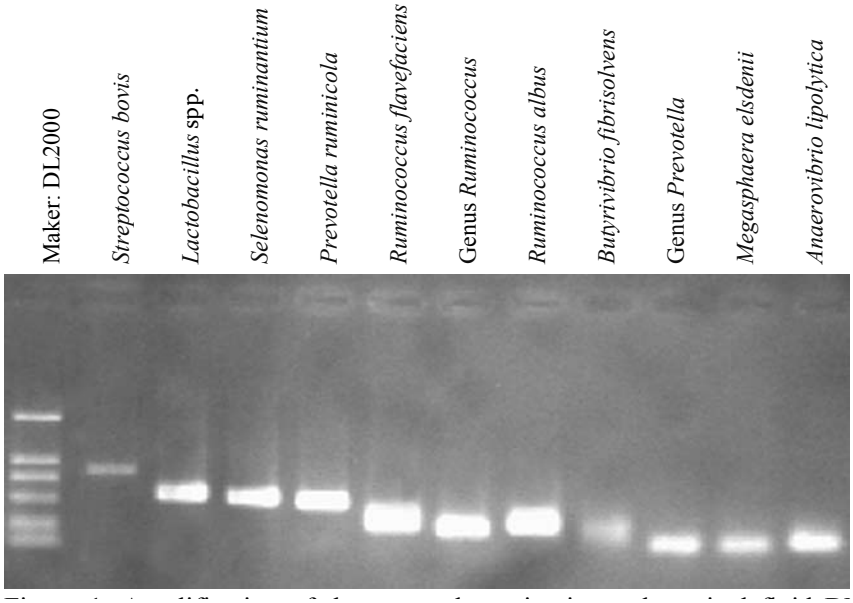

Figure 1. Amplification of the targeted species in total ruminal fluid DNA; DNA size marker (DL2000) is in the far left lane

7 min. PCR products were purified using the PCR clean-up kit (Takara, Japan).

The purified products were ligated into the pMD19 T-vector and then transformed into competent *Escherichia coli* DH5α cells (Takara, Japan). Positive transformants were randomly picked up and the recombinant plasmids were extracted using the standard alkaline lysis miniprep method (Sambrook et al., 1989). Positive recombinant plasmids were identified by PCR using T-vector universal primers: RV-M (5'-GAGCGGATAATTTCACACAGG-3') and M13-47 (5'-CGCCAGGGTTTTCCCAGTCACGA-3'). Verified positive recombinant plasmids were sequenced using the sequencer ABI 3730Xl (Applied Biosystems, USA).

Real-time PCR assays

DNA from each animal was used for real-time PCR quantification. Plasmid DNA containing the cloned target sequence was used as the standard DNA in real-time PCR and the target sequence was obtained by PCR cloning from rumen DNA according to Koike et al. (2007). After the confirmation of a single band of the correct size (Table 2) on an agarose gel (Figure 1), the PCR products were excised and purified using the Agarose Gel DNA Purification Kit (Takara, Japan), and then the pCR2.1 vector was ligated (Invitrogen, Japan). The ligation products transformed competent *Escherichia coli* DH5α cells. Plasmids were purified from transformed *E. coli* using a QIAprep Spin Miniprep Kit (Qiagen, USA) and the plasmids containing the correct insert were confirmed by PCR amplification of the target sequence and aligning the sequence against the GenBank database. The concentration of the plasmid was determined with NanoDrop 1000 (Thermo Scientific, German). Tenfold dilution series ranging from 1 to 10^9 copies were prepared for each target.

Real-time PCR quantification was performed in an ABI PRISM 7500HT Sequence Detection system (Applied Biosystems, USA). The reaction mixture (10 µl) was composed of 10mM Tris-HCl (pH 8.3), 50mM KCl, 1.5mM $MgCl_2$, 200µM (each) deoxynucleoside triphosphates, a 1:100 000 dilution of SYBR Green I (Molecular Probes, Eugene, Oreg.), 0.05U of *Taq* DNA polymerase (Takara, Tokyo, Japan) per µl, 0.25µM (each) specific primers, and 1 µl of × 1, × 10, and × 100 diluted template DNA. The amplification program consisted

Table 2. PCR primers for detection of rumen bacteria

Target bacterium	Primer sequences		Annealing temp (°C)	Product size (bp)	Reference
	forward	reverse			
Selenomonas ruminantium	TGCTAATACCGAATGTTG	TCCTGCACTCAAGAAAGA	53	513	Tajima (2001)
Ruminococcus albus	GTTTTAGGATTGTAAACCTCTGTCTT	CCTAATATCTACGCATTTCACCGC	60	270	BU et al. (2008)
Anaerovibrio lipolytica	AGACACGGCCCAAACTCCTACG	TCCTCCTTGACCCATTTCCTGA	60	157	BU et al. (2008)
Streptococcus bovis	CTAATACCGCATAACAGCAT	AGAAACTTCCTATCTCTAGG	57	869	Tajima (2001)
Genus *Prevotella*	GGTTCTGAGAGGAAGGTCCCC	TCCTGCACGCTACTTGGCTG	60		Stevenson and Weimer (2007)
Butyrivibrio fibrisolvens	GTTCGGCTGGGCACTCT	CACCGCGACATTCTGATTC	60	240	BU et al. (2008)
Lactobacillus spp.	GGA AAC AG(A/G) TGC TAA TAC CG	CAC CGC TAC ACA TGG AG	57	600	Heilig HG (2002)
Ruminococcus flavefaciens	GATGCCGCGTGGAGGAAGAAG	CATTTCACCGCTACACCAGGAA	60	286	BU et al. (2008)
Prevotella ruminicola	GGTTATCTTGAGTGAGTT	CTGATGGCAACTAAAGAA	53	485	Tajima (2001)
Genus *Ruminococcus*	GAGTGAAGTAGAGGTAAGCGGAATTC	GCCGTACTCCCCAGGTGG	60		Weimer et al. (2008)
Megasphaera elsdenii	GATTCTGGCTCAGGATGAACG	CGGGTGCTICCCACTTTCATG	60	128	Ouwerkerk (2002)

Table 3. Ruminal pH, VFA, L-lactic acid and ammonia-N concentration in steers fed a control diet or control diet supplemented with virginiamycin

	Diets		SEM	*P*
	control	treatment		
Ammonia N(mg/100 ml)	6.19	4.94	1.323	< 0.001
pH	6.63	6.70	0.145	0.040
Acetic (mmol/l)	53.71	52.17	4.192	0.270
Propionic (mmol/l)	13.72	12.82	1.336	0.100
Butyric (mmol/l)	17.71	21.05	2.429	< 0.001
Isovaleric (mmol/l)	1.26	1.36	0.214	0.500
Valeric (mmol/l)	0.95	0.97	0.080	0.640
Total VFA (mmol/l)	86.93	87.37	7.846	0.860
Acetic:Propionic	4.04	4.17	0.079	0.210
L-lactic acid (mmol/l)	1.39	1.26	0.183	0.300
Protozoa (log)	5.07	5.09	0.013	0.300

of one cycle of 94°C for 10 min, then 40 cycles of 94°C for 20 s, various annealing temperature (Table 2) for 33 s, and 72°C for 60 s and finally one cycle of 94°C for 15 s. The fluorescent product was detected at the last step of each cycle. After amplification, the melting curves were obtained by slow heating at 0.2°C/s increments from 60 to 99°C to verify the specificity of the PCR. For determination of the number of target species present in each sample, the fluorescent signals detected from two or three serial dilutions in the linear range of the assay were averaged and compared to a standard curve generated with standard DNA in the same experiment.

Data analysis

The counts and quantifications data were transformed into logarithmic form and then all data were analyzed as a crossover using the MIXED procedure of SAS 9.0 (SAS, 2004). We assumed steer as a random effect, whereas sampling times, periods, sequences and treatments as fixed effects. For the statistical analysis of ruminal fluid characteristics (pH, VFA, ammonia-N) and microbial counts and sampling time, the treatments were added to the model and analyzed using repeated measures. The significance level was declared at $P < 0.05$ unless otherwise noted. Trends for significance were declared at $P = 0.05$ to 0.10.

RESULTS

Ruminal fermentation parameters

Average ruminal pH, VFA, protozoa numbers and ammonia N are shown in Table 3. The supplementation of VM increased ruminal pH from 6.63 in the control group to 6.70 in the supplemented group ($P < 0.05$). The concentration of ammonia N was higher ($P < 0.01$) in the control group (6.19 mg per 100 ml) than in VM group (4.94 mg/100 ml). The propionate concentration of VM group (12.82 mmol/l) was not different from that of control group (13.72 mmol/l), but there was a downward trend in VM group ($P = 0.10$). The concentration of butyric acid increased from 17.71 mg/100 ml in the control to 21.05 mg/100 ml in VM group ($P < 0.01$) (Table 3). Accordingly, the acetic acid to propionate ratio was generally higher but not statistically significant in VM group (4.17) as compared with the control group (4.04). The supplementation of VM changed the L-lactic acid concentration as the mean L-lactic acid concentration decreased from 1.39 mmol/l in the control group to 1.26 mmol/l in VM group ($P = 0.30$). There was not a significant difference in the counts of pro-

Table 4. Colony counts of ruminal microorganisms from steers fed a control diet or control diet supplemented with virginiamycin

	Diets		SEM	*P*
	control	treatment		
Amylolytic bacteria (log_{10}CFU/ml)	8.72	8.41	0.098	< 0.001
Proteolytic bacteria (log_{10}CFU/ml)	8.83	8.60	0.069	< 0.001
Cellulolytic bacteria (log_{10}CFU/ml)	9.59	9.56	0.045	0.440
Total viable bacteria (log_{10}CFU/ml)	10.91	10.87	0.102	0.360

tozoa ($P = 0.30$) and in the concentration of total VFA ($P = 0.86$) between the treatment and the control.

Microorganism counts

Steers supplemented with VM had lower ($P < 0.01$) amylolytic bacteria (8.41 vs. 8.72 log_{10} CFU/ml) and proteolytic bacteria (8.60 vs. 8.83 log_{10} CFU/ml) counts as compared to the control (Table 4). There was not a significant difference in the counts of total viable bacteria ($P = 0.36$) and cellulolytic bacteria ($P = 0.44$) between the treatment and the control.

Quantification of ruminal bacteria

Primers were selected to allow for the specific species analyses. The gel electrophoresis bands of samples after the PCR run were specific and had the expected size (Figure 1). The results of quantification are shown in Table 5. The numbers of the Gram-negative bacteria *Selenomonas ruminantium* (5.55 log_{10} copies per µl) and *Anaerovibrio lipolytica* (8.12 log_{10} copies per µl) were higher in the rumen fluid of VM group than in that of the control (4.46 log_{10} copies per µl and 7.93 log_{10} copies per µl, respectively). The numbers of the Gram-positive bacteria *Streptococcus bovis* (6.67 log_{10} copies per µl) and *Ruminococcus albus* (6.95 log_{10} copies per µl) were lower in the rumen of VM group than in that of the control (7.74 log_{10} copies per µl and 7.39 log_{10} copies per µl, respectively). VM supplementation led to an upward trend of the quantification of lactic acid-utilizing bacteria (*Selenomonas ruminantium* and *Anaerovibrio lipolytica*) and a downward trend of the quantification of lactic acid-producing bacteria (*Streptococcus bovis* and

Table 5. Quantification of ruminal bacteria by real-time PCR (log_{10} copies/µl) in steers fed a control diet or control diet supplemented with virginiamycin

	Diets		SEM	*P*
	control	treatment		
Selenomonas ruminantium	4.46	5.55	0.354	0.05
Ruminococcus albus	7.39	6.95	0.165	0.07
Anaerovibrio lipolytica	7.93	8.12	0.120	0.09
Streptococcus bovis	7.74	6.67	0.420	0.10
Genus *Prevotella*	10.04	9.99	0.029	0.25
Butyrivibrio fibrisolvens	4.74	4.44	0.215	0.35
Lactobacillus spp.	5.22	4.57	0.518	0.41
Ruminococcus flavefaciens	4.00	3.77	0.245	0.52
Prevotella ruminicola	4.32	4.79	0.482	0.54
Genus *Ruminococcus*	8.45	8.36	0.141	0.67
Megasphaera elsdenii	2.27	2.26	0.231	0.97

Lactobacillus spp.). The supplementation of VM decreased the relative abundance of *Lactobacillus* spp., but not statistically significantly. The quantifications of the genus *Prevotella*, *Prevotella ruminicola*, *Butyrivibrio fibrisolvens*, genus *Ruminococcus*, *Ruminococcus flavefaciens* and *Megasphaera elsdenii* were not affected by VM.

DISCUSSION

The rumen pH was higher in steers fed VM than in those that did not receive any VM. This result is consistent with the study by Hedde et al. (1980), who reported a higher rumen pH with VM supplementation than in the non-medicated controls. Clayton et al. (1999) also found that cows fed VM tended to have a higher ruminal pH than those without VM.

As VM has selective effects on ruminal bacteria, VM has the potential to moderate and stabilize ruminal fermentation (Coe et al., 1999). The effect of VM on ruminal N metabolism has been studied to a much lesser extent. In our study, the ruminal ammonia-N concentration was lower due to VM supplementation. The numerical decrease in rumen ammonia might hint VM spared amino acids from degradation in the rumen. *In vitro* studies by Van Nevel et al. (1984) and Van Nevel and Demeyer (1990) reported that the effects of VM were similar to those of monensin *in vitro* (i.e. reduction in casein degradation and ammonia production). Therefore we think that VM reduced the ruminal ammonia-N concentration by inhibiting the activity of the same hyper ammonia-producing bacteria that were sensitive to monensin. This group of bacteria was originally identified by Russell et al. (1988) and characterized as having a high deaminative activity and as being responsible for a significant proportion of ammonia produced in the rumen.

Ruminal fermentation was not affected by VM supplementation as indicated by changes in total VFA, propionate, valerate and isovalerate concentrations. Our results (except for butyrate) were consistent with several studies (Hedde et al., 1983; Zinn, 1987; Morris et al., 1990; Coe et al., 1999; Valentine et al., 2000; Ives et al., 2002) indicating that the addition of VM had no effect on either the concentration of total volatile fatty acids in the rumen or the proportions of the individual volatile fatty acids (Coe et al., 1999; Valentine et al., 2000). Other studies have reported that VM increases the proportion of propionate relative to other VFA (Hedde et al., 1983; Nagaraja et al., 1995a,b). This effect of VM was not observed in our experiment, and it might be due to the increased absorption of additional propionate or factors other than VFA production in the animal (Clayton et al., 1999). The higher concentrations of butyrate in VM-treated steers could be due to organisms like *Megasphaera elsdenii*, an active lactate utilizer in the rumen that is not inhibited by monensin, tylosin, and VM (Nagaraja and Taylor, 1987). Numerous experiments have shown reduced lactate production in rumen fluid *in vitro* and *in vivo* (Hedde et al., 1982; Nagaraja et al., 1987; McDonald et al., 1994; Clayton et al., 1999). In our study, VM decreased the L-lactic acid concentration, but not statistically significantly. Although a reduction in lactate with VM (Clayton et al., 1999) was expected, our studies with induced subacute acidosis did not detect any significant differences in the ruminal lactate concentration as did Brown et al. (2000).

It is believed to alter ruminal fermentation primarily by changing ruminal microbial populations (Ives et al., 2002). VM has an antimicrobial spectrum similar to that of monensin: Gram-positive bacteria are susceptible and Gram-negative bacteria are generally resistant (Nagaraja and Taylor, 1987). The antimicrobial activity and subsequent alterations in ruminal fermentation products are similar to those of monensin (Hedde et al., 1982; Nagaraja et al., 1997).

In the present study, steers fed supplemental VM had the lower amylolytic bacteria and proteolytic bacteria count which might accounted for the VM as a potent inhibitor of amylolytic bacteria and proteolytic bacteria. The proportion of amylolytic bacteria in the rumen can be as high as 90% to 95% of total culturable bacteria in grain-fed animals (Leedle and Hespell, 1980). Nagaraja and Titgemeyer (2007) believed that ruminal bacteria respond to increased availability of fermentable substrates by increasing growth rates and fermentative activities, which leads to the increased production of ruminal fermentation. In our study, the supplementation of VM to the diet inhibited the activity of amylolytic bacteria and proteolytic bacteria, which resulted in an increase in ruminal pH and a decrease in L-lactic acid accumulation. The counts of total viable bacteria, cellulolytic bacteria and protozoa did not show any differences between the treatment and the control. Several studies (Coe et al.,

1999; Candanosa et al., 2008) indicated that VM had not effect on rumen ciliates.

The extreme complexity of the ruminal microbiota has been uncovered in numerous publications that employed the isolation of pure cultures. The techniques of molecular microbial ecology provide an opportunity to quantify these ruminal species with great sensitivity and precision. Real-time PCR was used in several studies to quantify the abundance of particular bacterial species in the rumen under a variety of conditions (Tajima et al., 2001; Klieve et al., 2003). In this study, we quantified the shifts in populations of a number of well-characterized ruminal bacterial species based on the hypothesis that at least some species should respond to the VM inclusion in and its subsequent withdrawal from the diet. The populations of individual species and genera of bacteria were determined using an absolute quantification procedure (Koike et al., 2007). Several studies (Muir and Barreto, 1979; Dutta and Devriese, 1981; Nagaraja and Taylor, 1987) indicated that VM is a potent inhibitor of ruminal lactic acid-producing bacteria. In this study, we found that the counts of *Streptococcus bovis* and *Lactobacillus spp.* were decreased, which is consistent with those reports. *Selenomonas ruminantium*, which can contribute to both lactic acid production and utilization (Nagaraja and Titgemeyer, 2007), had an increasing trend in VM supplemented diet. *Anaerovibrio lipolytica* also had an increasing trend in VM supplemented diet. *Anaerovibrio lipolytica* and *Megasphaera elsdenii* are known to ferment lactic acid in ruminal bacterial species (Nagaraja and Titgemeyer, 2007). It is estimated that *Megasphaera elsdenii* ferments 60 to 80% of the DL-lactate in the rumen (Counotte et al., 1981). However, *Megasphaera elsdenii* is not inhibited by VM (Nagaraja and Taylor, 1987). VM was inhibitory to Gram-positive bacteria and to those bacteria that often stain as Gram-negative ones but have the Gram-positive type of cell wall structure (Paterson et al., 1975; Cheng and Costerton, 1977; Nagaraja and Taylor, 1987). In this study, the Gram-positive bacteria *Streptococcus bovis*, *Lactobacillus* spp., *Ruminococcus flavefaciens* and *Butyrivibrio fibrisolvens* were lower in the VM treatment than in the control, but the difference was not significant. However, these trends were in agreement with the Gram-positive bacteria inhibition and showed that VM has selective effects on ruminal bacteria and influences ruminal fermentation by changing a part of the specific ruminal bacteria populations.

The basal diets were designed to increase the amount of readily fermentable feedstuffs and to reduce pH in the rumen. The reduction in ruminal pH in the control group after the experimental diet was introduced is consistent with the rapid fermentation of a grain-based diet with a high content of starch. However, the ruminal pH of all steers was relatively high throughout the trial. The diet did not provide any clinical signs of acute or subacute acidosis (Garrett, 1996) in any steers during this study. Even if this was the case, important observations can still be made about relative differences between groups with respect to pH, ammonia, VFA and ruminal microorganisms.

CONCLUSIONS

Virginiamycin in diets led to an increase in ruminal pH, ruminal ammonia utilization and butyrate concentration, and reduced the risk of lactic acidosis in steers. Moreover, the data demonstrated that VM has selective effects on ruminal bacteria and influences ruminal fermentation by changing a part of the specific ruminal bacteria populations.

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