APPLIED AND ENVIRONMENTAL MICROBIOLOGY, July 1983, p. 160–164
0099-2240/83/070160-05$02.00/0

Vol. 46, No. 1

Monensin-Resistant Bacteria in the Rumens of Calves on Monensin-Containing and Unmedicated Diets†

K. A. DAWSON* AND J. A. BOLING

Department of Animal Sciences, University of Kentucky, Lexington, Kentucky 40546-0215

Received 31 January 1983/Accepted 29 April 1983

Total and monensin-resistant anaerobic bacterial populations and volatile fatty acid concentrations were examined in the rumens of steers fed monensin-containing (33 mg/kg) and unmedicated diets. Total anaerobic counts on a habitat-simulating medium ranged from 7.1×10^8 to 7.1×10^9 CFU/g of rumen ingesta and were not significantly different in animals fed the two diets. The mean percentage of the anaerobic population resistant to monensin (10 μg/ml) was significantly greater in animals receiving the monensin-supplemented diet for 33 days than in those receiving the unmedicated diet (63.6 and 32.8%, respectively). Treatment group differences in monensin resistance tended to develop later than characteristic differences in acetate/propionate ratios. Relative proportions of resistant organisms in monensin-fed animals remained significantly greater for at least 18 days after monensin was deleted from the ration, whereas acetate/propionate ratios increased to values comparable to those in the control within 10 days. These data suggest that monensin-resistant bacteria may be present in greater numbers in the rumens of animals fed monensin-supplemented diets. However, greater proportions of monensin-resistant organisms were not necessarily associated with altered fermentation patterns.

The effects of the polyether antibiotic monensin on animal performance and rumen fermentations have been well documented (7, 12–18). It is generally believed that the growth-promoting activity of monensin can be explained in part by altered microbial activity in the rumen. However, there is relatively little information on the effects of monensin and similar growth-promoting ionophores on the composition of the microbial population in the rumen.

Recent in vitro studies of representative rumen isolates have shown that hydrogen-, formate-, acetate-, butyrate-, and lactate-producing bacteria tend to be inhibited by monensin, whereas succinate- and propionate-producing bacteria and lactate-utilizing organisms are resistant to or rapidly develop resistance to monensin (4, 6, 9). As a result of these studies, Chen and Wolin (4) have suggested a model in which monensin-induced changes in ruminal fermentations can be explained by the selective enrichment of succinate and propionate producers and the inhibition of monensin-sensitive hydrogen, formate, and butyrate producers. Few studies have examined the selection of monensin resistance in microbial populations within the rumen. Studies by Nagaraja and his associates (11) have shown the selective inhibition of streptococci and lactobacilli in the rumens of monesin-fed animals, whereas Brulla and Bryant (Abstr. Annu. Meet. Am. Soc. Microbiol. 1980, I121, p. 104) have shown increased proportions of *Bacteroides* species and a decreased proportion of monensin-sensitive *Butyrivibrio fibrisolvens* in the rumens of animals fed monensin. The observations in these studies are consistent with the hypothesis that monensin-resistant bacteria are selected in the rumen.

In addition to predicting the selection of monensin-resistant strains in the rumen, the results obtained from the model described here suggest that the selection of resistant strains in the rumen is closely correlated with changes in ruminal fermentations which are characteristically associated with monensin supplementation. In this study we examined the proportions of monensin-resistant bacteria and the fermentation patterns in the rumens of calves fed monensin-containing and unmedicated diets to determine whether the selection of monensin-resistant bacteria could account for the changes in ruminal fermentations observed when monensin is included in the diet.

MATERIALS AND METHODS

Treatment groups. Six Angus heifers weighing between 315 and 355 kg were fitted with rumen cannulae

† Paper no. 82-5-229 of the Kentucky Agricultural Experiment Station.

and randomly assigned to treatment groups. Three animals were assigned to each of two treatment groups. All animals were fed 3 kg of a basal diet containing 20% (wt/wt) cottonseed hulls, 11% soybean meal, 68% cracked corn, and 1% trace mineral salts twice daily. Animals in the control group received the unmedicated diet throughout the 141-day study period. Animals in the monensin treatment group received the same unmedicated diet during a 34-day adjustment period and then were adapted over a 24-h period to a diet containing 33 mg of monensin per kg of basal diet. The monensin treatment group received the monensin-containing diet throughout a 33-day treatment period and was returned to an unmedicated diet for a 73-day posttreatment period. The animals were housed in individual concrete-floored pens designed to prevent direct contact among animals.

Sample collection. Rumen fluid samples were collected via cannulae with a 50-ml catheter syringe and a Tygon tube (inside diameter, 5 mm; length, 1 m) attached to a stainless steel suction strainer (1.2-mm hole diameter; Precision Machine Co., Lincoln, Nebr.) 1 to 2 h after the morning feeding. The first two aliquots from each steer were discarded to minimize oxygen exposure in subsequent aliquots. Each sample was a composite of at least four aliquots. The sampling tube was adjusted after the collection of each aliquot to allow for collection at several points within the rumen. Samples were maintained in a 38°C water bath and were transported to the microbiology laboratory in screw-capped bottles which had been filled to capacity. Sample dilutions were prepared in an anaerobic chamber (Coy Laboratory Products, Ann Arbor, Mich.) containing an atmosphere of 10% CO_2–5% H_2–85% N_2. Samples of the rumen fluid (11 ml each) were diluted in 99 ml of anaerobic dilution solution (3) in blender jars. The initial dilution was blended at the maximum speed for 2 min. Serial dilutions were sealed in culture tubes and removed from the chamber to inoculate anaerobic roll tubes. An undiluted aliquot was frozen for subsequent volatile fatty acid (VFA) analysis. pH measurements were made on a second undiluted aliquot. The preparation of samples for microbial and VFA analysis was complete within 15 min after samples were taken from the rumen.

Samples for VFA, pH, and microbial analyses were collected twice during the adjustment period (1 and 3 weeks before treatment) four times during the treatment period (at approximately weekly intervals), and four times during the posttreatment period.

Enumeration of bacteria. All media were prepared and inoculated under a CO_2 gas phase by using a modification of the Hungate technique (2). Total anaerobic populations were enumerated in five replicate roll tubes (18 by 150 mm) containing 7 ml of CCA medium (1) prepared with energy-depleted rumen fluid (5). Monensin-resistant organisms were enumerated on medium prepared by adding 10 μg of crystalline monensin per ml in a stock solution (10 mg/ml) in denatured ethanol. Each of the five tubes in the replicate set was inoculated with 0.1 ml of the appropriate dilution of rumen contents. CFU were counted after 5 days of incubation at 38°C. Mean counts for each sample represent the mean values obtained from the three tubes showing the greatest number of CFU in each replicate set. Replicate sets with between 30 and 300 colonies per tube were counted. This procedure provided a standard method for eliminating the great variability observed when tubes which were inadvertently oxidized were used to calculate the mean count. Percentages of monensin-resistant organisms were determined by dividing the mean counts obtained on the monensin-supplemented medium by the mean counts obtained on the similar antibiotic-free medium and multiplying by 100.

VFA analysis. An undiluted subsample of each rumen fluid sample was thawed and subjected to VFA analysis on a Varian model 2100 gas chromatograph equipped with a flame ionization detector (Varian Instrument Division, Palo Alto, Calif.) by using a modification of the methods described by Erwin et al. (8). VFAs were quantitatively separated on a glass column (inside diameter, 2 mm; length, 1.83 m) packed with 10% SP-1200–1% H_3PO_4 on 80/100 Chromosorb WAW (Supelco, Inc., Bellefonte, Pa.) at 125°C, using cyclohexanone as an internal standard. Total VFA concentrations were calculated from the three predominant acids present and represent the total of the acetate, propionate, and butyrate concentrations. Valerate, isovalerate, and isobutyrate concentrations consistently accounted for less than 2% of the VFAs present and were not significantly influenced by monensin treatment.

Statistical analysis. Treatment differences during the treatment and posttreatment periods were analyzed with the Tables *t*-test procedures provided in the current Statistical Analysis System (10). Mean colony counts were subjected to $\log_{10}$ transformation before statistical analysis. Each value shown in the figures represents the mean of three animal observations during the treatment and posttreatment periods and the mean of six animal observations during the adjustment period.

RESULTS AND DISCUSSION

Characteristics of the rumen fermentations. The mean total VFA concentration in the rumens of control calves decreased during the adjustment period and the early part of the treatment period (Fig. 1). Decreases in acid concentrations during these periods apparently reflected natural variations in the rumens, since these animals were not subject to changes in diet. Total VFA concentrations were not found to be different ($P > 0.10$) in the two treatment groups during the adjustment period. The mean total VFA concentrations in monensin-fed animals were found to be greater ($P < 0.10$) than those in the control animals during the entire treatment period. However, VFA concentrations in monensin-fed animals were not sigificantly greater ($P > 0.10$) during the posttreatment period and returned to values comparable to those in the control animals within 10 days after monensin was removed from the diet. Increases in ruminal VFA concentrations have been reported in cattle fed low levels of monensin in concentrate rations (17), whereas other studies have shown that total VFA concentra-

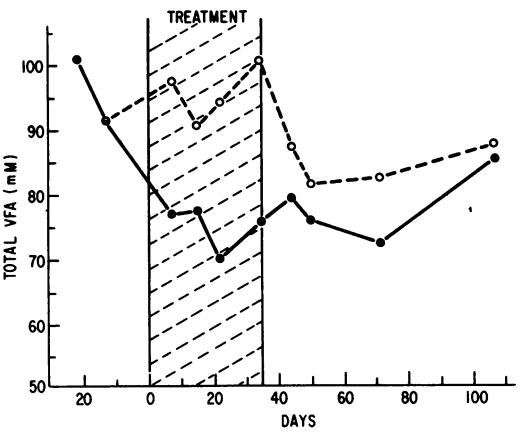

FIG. 1. Total VFA concentrations in rumen fluids of calves fed monensin-containing (○) and unmedicated (●) diets. Pretreatment values are the means collected from six animals, whereas treatment and posttreatment values represent the means collected from three animals in each treatment group.

tions are not affected by monensin supplementation (7).

The mean pH value for the rumens of animals on the control diet was 6.4 during the treatment period. The mean pH values for the rumens of monensin-fed animals were not significantly different but were consistently 0.1 to 0.3 units lower than that in the control animals during the treatment period.

The ratios of acetate concentration to propionate concentration were also found to be variable in animals fed the unmedicated diet and tended to increase from a mean value of 2.50 during the adjustment period to a mean value of 4.50 at the end of the posttreatment period (Fig. 2). However, acetate/propionate ratios were significantly lower ($P < 0.10$) in the monensin-fed animals during sampling periods within the treatment period. The ratios in the monensin treatment group returned to values similar to those in the control group within 10 days after monensin was removed from the diet. Both in vitro and in vivo studies have shown that propionate concentrations are increased, whereas acetate and butyrate concentrations are decreased, in the rumens of monensin-fed cattle (7, 12–16, 18). Many investigators have used the ratio of acetate concentration to propionate concentration as an indicator of the effects of monensin on ruminal fermentations (7, 16). In our study, decreased acetate/propionate ratios in monensin-fed animals were only observed during the treatment period. Our data suggest that both increased VFA concentrations and decreased acetate/propionate ratios were directly associated with monensin supplementation.

Monensin-resistant bacteria. The mean total viable count in the rumens of calves in both treatment groups ranged from 7.1×10^8 to 7.1×10^9 CFU/g of rumen ingesta during this study. The mean total counts varied greatly from one sampling period to the next but were not found to be significantly altered ($P > 0.10$) by monensin supplementation. This observation is consistent with those of other researchers (7), who have reported that monensin has no significant effects on the total bacterial concentration in the rumen.

The proportions of the microbial population that grew on media contianing 2.5, 5.0, and 10.0 μg of monensin per ml were examined in two preliminary experiments with steers fed unmedicated diets. The mean percentages of the ruminal populations recovered on these media were 53, 47, and 46%, respectively, and were not found to be significantly different. A monensin concentration of 10.0 μg/ml was selected to enumerate resistant bacteria in the subsequent studies.

The mean percentage of the bacterial population resistant to 10 μg of monensin per ml in samples taken during the adjustment period was 51% in the control group and 56% in the monensin treatment group. During this period, percentages ranged from 42 to 63% for individual animals and were not significantly different ($P > 0.10$) between the two treatment groups. These data suggest a similar distribution of resistant organisms in the two treatment groups before the initiation of dietary treatments.

There was considerable temporal variation in the proportions of bacteria from control animals

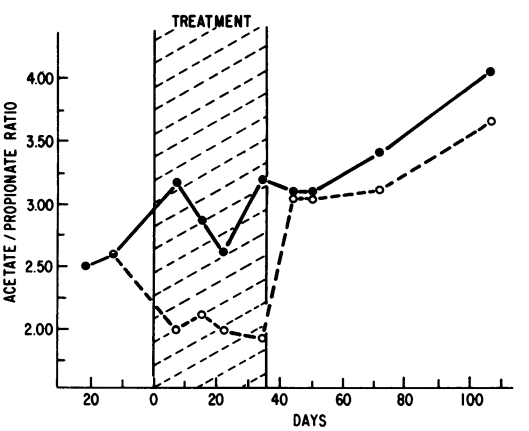

FIG. 2. Ratios of acetate concentration to propionate concentration in the rumens of calves fed monensin-containing (○) and unmedicated (●) diets. Pretreatment values are the means collected from six animals, whereas treatment and posttreatment values represent the means collected from three animals in each treatment group.

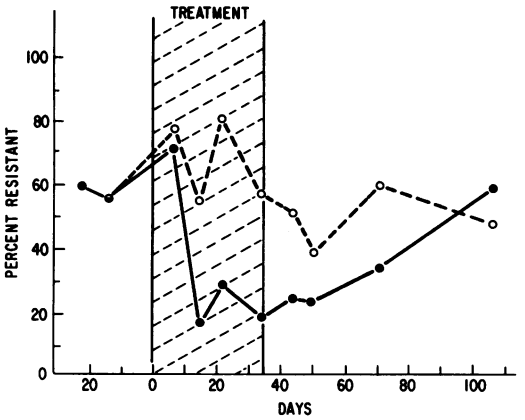

FIG. 3. Percentage of the total microbial population resistant to 10 μg of monensin per ml in the rumens of calves fed monensin-containing (○) and unmedicated (●) diets. Pretreatment values are the means collected from six animals, whereas treatment and posttreatment values represent the means collected from three animals in each treatment group.

resistant to 10 μg of monensin per ml during the treatment and posttreatment periods (Fig. 3). The mean percentages of monensin-resistant bacteria were not significantly different ($P > 0.10$) in the two treatment groups during the first 8 days of the treatment period, but were greater ($P < 0.10$) in the monensin-fed calves after day 14 of the treatment period. The peak difference in resistance levels was reached after 24 days of treatment when the mean percentage in monensin-fed animals was 50 percentage units greater than that observed in the controls and 25 percentage units greater than that observed in the same calves during the adjustment period. The proportion of resistant bacteria in the monensin-fed group remained greater ($P < 0.10$) for more than 18 days after monensin was removed from the diet.

The differences in the distribution of monensin-resistant organisms in the two treatment groups reflects the selective activity of monensin in the rumen, since similar distribution differences were not observed during the adjustment period. These data are consistent with the hypothesis that monensin supplementation results in the selection of monensin-resistant organisms in the rumen (4, 9). However, greater levels of resistance in monensin-fed animals were only apparent when measured relative to those in the within-sampling-period controls. The proportions of resistant bacteria in monensin-fed animals during the treatment and posttreatment peirods were not consistently greater than those observed during the adjustment period. Therefore, it was not possible to show a selective increase in the proportions of resistant bacteria in monensin-fed animals during the course of our study.

Treatment differences in monensin resistance were not correlated with differences in total VFA concentrations or the characteristic differences in acetate/propionate ratios. Significant treatment differences in resistance were not measured immediately after monensin was included in the diet and were present for more than 18 days after monensin was removed from the diet. In contrast, differences in acetate/propionate ratios and total VFA production were only noted during the period when monensin was included in the diet. Our data suggest that the metabolic changes that occur in the rumen are not associated with increased proportions of monensin-resistant bacteria and that increased monensin resistance may not be reflected in ruminal fermentation patterns. These observations do not fit with the selection model developed from in vitro studies of rumen bacteria (4, 9) and suggest that the selection of monensin-resistant microbial groups within the rumen cannot completely account for changes in ruminal fermentations or the growth-promoting activity of these antibiotics.

ACKNOWLEDGMENTS

We are grateful to Wanda S. Cain and Calvin M. Willis for their excellent technical assistance during this study.

This research was supported by the Agricultural Experiment Station of the University of Kentucky.

LITERATURE CITED

1. **Allison, M. J., I. M. Robinson, J. A. Bucklin, and G. D. Booth.** 1979. Comparison of bacterial populations of the pig cecum and colon based upon enumeration with specific energy sources. Appl. Environ. Microbiol. **37**:1142–1151.
2. **Bryant, M. P.** 1972. Commentary on the Hungate technique for culture of anaerobic bacteria. Am. J. Clin. Nutr. **25**:1324–1328.
3. **Bryant, M. P., and I. M. Robinson.** 1961. An improved nonselective culture medium for ruminal bacteria and its use in determining diurnal variation in numbers of bacteria in the rumen. J. Dairy Sci. **44**:1446–1456.
4. **Chen, M., and M. J. Wolin.** 1979. Effect of monensin and lasalocid-sodium on the growth of methanogenic and rumen saccharolytic bacteria. Appl. Environ. Microbiol. **38**:72–77.
5. **Dehority, B. A., and J. A. Grubb.** 1976. Basal medium for the selective enumeration of rumen bacteria utilizing specific energy sources. Appl. Environ. Microbiol. **32**:703–710.
6. **Dennis, S. M., T. G. Nagaraja, and E. E. Bartley.** 1981. Effects of lasalocid or monensin on lactate-producing or -using rumen bacteria. J. Anim. Sci. **52**:418–426.
7. **Dinius, D. A., M. E. Simpson, and P. B. Marsh.** 1976. Effect of monensin fed with forage on digestion and the ruminal ecosystem of steers. J. Anim. Sci. **42**:229–234.
8. **Erwin, E. S., G. J. Marco, and E. M. Emery.** 1961. Volatile fatty acid analyses of blood and rumen fluid by gas chromatography. J. Dairy Sci. **44**:1768–1771.
9. **Henderson, C., C. S. Stewart, and F. V. Nekrep.** 1981. The effect of monensin on pure and mixed cultures of rumen bacteria. J. Appl. Bacteriol. **51**:159–169.
10. **Hewlwig, J. T., and K. A. Council.** 1979. Statistical Anal-

ysis System user's guide. Statistical Analysis System Institute Inc., Cary, N.C.
11. **Nagaraja, T. G., T. B. Avery, E. E. Bartley, S. K. Roof, and A. D. Dayton.** 1982. Effect of lasalocid, monensin or thiopeptin on lactic acidosis in cattle. J. Anim. Sci. **54**:649–658.
12. **Potter, E. L., C. O. Cooley, L. F. Richardson, A. P. Raun, and R. P. Rathmacher.** 1976. Effect of monensin on performance of cattle fed forage. J. Anim. Sci. **43**:665–669.
13. **Prange, R. W., C. L. Davis, and J. H. Clark.** 1978. Propionate production in the rumen of Holstein steers fed either a control or monensin supplemented diet. J. Anim. Sci. **46**:1120–1124.
14. **Raun, A. P., C. O. Cooley, E. L. Potter, R. P. Rathmacher, and L. F. Richardson.** 1976. Effect of monensin on feed efficiency of feedlot cattle. J. Anim. Sci. **43**:670–677.
15. **Richardson, L. F., A. P. Raun, E. L. Potter, C. O. Cooley, and R. P. Rathmacher.** 1976. Effect of monensin on rumen fermentation in vitro and in vivo. J. Anim. Sci. **43**:657–664.
16. **Slyter, L. L.** 1979. Monensin and dichloroacetamide influences on methane and volatile fatty acid production by rumen bacteria in vitro. Appl. Environ. Microbiol. **37**:283–288.
17. **Thornton, J. H., and F. N. Owens.** 1981. Monensin supplementation and in vivo methane production by steers. J. Anim. Sci. **52**:628–634.
18. **Van Nevel, C. J., and D. I. Demeyer.** 1977. Effect of monensin on rumen metabolism in vitro. Appl. Environ. Microbiol. **34**:251–257.