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Methane Production by Fermentor Cultures Acclimated to Waste from Cattle Fed Monensin, Lasalocid, Salinomycin, or Avoparcin

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The ability of microorganisms to ferment waste from cattle fed monensin, lasalocid, or salinomycin to methane was determined. Continuously mixed anaerobic fermentors with 3-liter working volumes at 55°C were used; fermentors were fed once per day. Initially, all fermentors were fed waste without antibiotics at 6% volatile solids (VSs, organic matter) and a 20-day retention time (RT) for 60 days. Waste from animals fed monensin, lasalocid, or salinomycin at 29, 20, and 16.5 mg per kg of feed, respectively, was added to duplicate fermentors at the above VSs, and RT. Avoparcin (5 to 45 mg/liter) was not fed to animals but was added directly to duplicate fermentors. Lasalocid and salinomycin had minimal effects on the rate of methane production at RTs of 20 days and later at 6.5 days. Avoparcin caused an increase in organic acids from 599 to 1,672 mg/liter (as acetate) after 4 weeks, but by 6 weeks, acid concentrations declined and the rate of methane production was similar to controls at a 6.5-day RT. The monensin fermentors stopped producing methane 3 weeks after antibiotic addition. However, after a 6-month acclimation period, the microorganisms apparently adapted, and methane production rates of 1.65 and 2.51 liters per liter of fermentor volume per day were obtained with 6% VSs, and RTs of 10 and 6.5 days, respectively. This compares with 1.78 and 2.62 liters/liter per day for controls ($P > 0.05$). All fermentors that were fed waste containing antibiotics had lower pH values and ammonia and alkalinity concentrations, suggesting less buffering capacity and protein catabolism than in controls. Acclimation results obtained with fermentors at 35°C were similar to those for fermentors at 55°C. These studies indicate that waste from cattle fed these selected growth-promoting antibiotics can be thermophilically fermented to methane at RTs of 6.5 days or longer and VS concentrations of 6%, at rates comparable to waste without antibiotics.

The effect of dietary growth promoters and their metabolites on the anaerobic fermentation of animal wastes to methane has received little attention. Iannotti and Fischer (Agric. Wastes, in press) studied the short-term effect (4-day duration) of 18 antibiotics and feed additives on the anaerobic fermentation of swine manure to methane at 35°C. Most of these compounds did not affect, or only temporarily reduced, biogas production when growth-promoting levels were added. At higher concentrations, the majority reduced methane production, although few completely inhibited it.

Brumm and Sutton (3) found that concentrations of copper sulfate greater than 50 mg/liter inhibit the decomposition of swine waste in anaerobic pits. Brumm et al. (5) reported that dietary arsonic acids caused increased dry matter and volatile solid (VS) destruction of this same waste in anaerobic pits. This was also shown to be true in swine waste digesters which contain arsonic acids (4). However, the altered digestion, i.e., increased dry matter and VS destruction, is apparently not accompanied by increased methane generation but instead results in the increased conversion of organics to volatile fatty acids (VFAs).

Simulating a rumen fermentation using continuous-flow fermentors, Fuller and Johnson (8) determined the effects of monensin and lasalocid on the efficiency of fermentation of high grain or roughage substrates. Methane production was significantly depressed by the ionophore additions from both substrates. Individual VFA patterns were generally shifted without affecting total VFA production. Substrate nitrogen digested and the resulting concentration of total effluent nitrogen and ammonia were significantly depressed as a result of ionophore supplementation. Our previous results from methane-produc-

ing fermentors which were fed waste from cattle receiving dietary supplements of monensin indicated that methane production was completely inhibited at 35 and 55°C (18).

The present study was initiated to determine whether the microorganisms in methane-producing fermentors could be acclimated to and effectively ferment waste from beef cattle which received dietary supplements of monensin, lasalocid, or salinomycin. Avoparcin was also studied; however, it was added directly to the fermentors. The results indicate that the microorganisms will adapt to the antibiotics.

MATERIALS AND METHODS

Fermentation vessels used in the study were 4-liter Pyrex aspirator bottles which have been previously described (18, 20).

Waste was collected from 250- to 270-kg steers fed ad libitum either a basal diet (dry matter basis) of 87.9% corn, 7.0% corn silage, 3.25% soybean meal, and a vitamin-mineral supplement or a basal diet plus (mg per kg of feed) monensin (Eli Lilly & Co.), 29; lasalocid (Hoffmann-La Roche Inc.), 20; or salinomycin (A. H. Robins Co.), 16.5. Avoparcin (American Cyanamid Co.) was added directly to fermentors beginning with 5 mg/liter and going up to 45 mg/liter. The concentrations of the growth promoters were within the range normally prescribed for beef cattle. Three steers (total of twelve) were assigned to each diet. The animals were confined to indoor metabolism stalls on concrete floors. After the animals were fed the diets for 21 days, waste (feces and urine) was collected daily and stored at 4°C until 200 kg was accumulated. The waste was dispensed into screw capped plastic bottles and stored at −20°C until 1 day before use, as described previously (20).

Methane production in fermentors fed once per day was initiated by adding 3.0% control waste (without antibiotics) plus a 20% inoculum from an established methane-producing fermentor. Duplicate fermentors per treatment were initially established with control waste at a loading rate of 6% VSs (organic matter) and a 20-day retention time (RT). After four volume turnovers or 80 days, each fermentor (total of 10) was sampled on 3 consecutive days. After sampling, the contents of all fermentors were intermixed before receiving the antibiotic-containing waste to insure that all fermentors contained similar microorganisms. Duplicate fermentors then received waste from animals fed either monensin, lasalocid, salinomycin, or no antibiotics at the above loading rate. Fermentors were sampled once per week. If no inhibition was observed due to the antibiotics, the RT was gradually descreased to 4 days while 6% VSs was maintained. Steady-state data (3 consecutive days after four volume turnovers) were collected at RTs of 10 and 6.5 days or as noted in the tables. Avoparcin was added to duplicate fermentors as follows (mg/liter): week 1, 5; week 2, 10; weeks 3 through 6, 15; weeks 7 and 8, 20; weeks 9 and 10, 25; weeks 11 and 12, 30; weeks 14 through 26, 45. The RT for the avoparcin fermentors was maintained at 20 days until the 14th week, after which it was gradually decreased to 4 days.

Gas volume was determined by syphon displacement of a 20% NaCl–0.5% citric acid solution as previously described (19). Total solids, VSs, and total volatile acids (salicic acid method) were measured by previously published methods (1). Individual VFAs were measured by methods described previously (9, 14, 19), as were Kjeldahl and ammonia nitrogen (20). The influent waste was adjusted to 6% VSs before VFA analyses began. VFAs in the fermentors were determined from samples of the effluent. Methane production and Kjeldahl and ammonia nitrogen data from steady-state fermentors and influent waste were compared by the Student's t test (16).

RESULTS

After repeated efforts to adapt microorganisms at 55°C to waste containing monensin, one fermentor was acclimated after a 6-month period. This fermentor initially received waste from cattle which were not fed antibiotics (control waste) and then waste from cattle fed monensin beginning with a 47-day RT and 6% VSs. VFAs and pH were determined periodically. If pH fell below 6.0, sodium hydroxide was added to maintain it at 7.0. The RT was gradually decreased over a 6-month period during which time a duplicate fermentor was started with inoculum from the acclimated fermentor. Steady-state data were collected from the acclimated fermentors at RTs of 10 and 6.5 days (Table 1). The data indicate that 1.65 compared with 1.78 liters of methane per liter of fermentor per day ($P > 0.05$) were produced for the monensin and control fermentors, respectively, at the 10-day RT. Later, rates of 2.51 and 2.62 liters of methane per liter of fermentor per day ($P > 0.05$) were obtained at a RT of 6.5 days for the control and monensin fermentors, respectively. The percent methane in the biogas for the fermentors fed the waste containing monensin was 2% less than in the controls. The concentration of organic acids was twofold greater in the monensin fermentors, while the Kjeldahl and ammonia nitrogen levels ($P < 0.05$) and pH were lower than in the control fermentors. Higher concentrations of propionate, 3.9 and 2.9 mM, at RTs of 10 and 6.5 days, respectively, were detected in the monensin fermentors compared with trace quantities (≤0.1 mM) found in the controls (Table 2). The fermentors fed waste which contained monensin failed at a 4-day RT (pH 5.8; total organic acids, 6,500 mg of acetate per liter); however, the controls were successfully maintained at this RT (pH 7.03; total organic acids, 1,559 mg of acetate per liter).

Table 3 shows a comparison of data from fermentors at 55°C which received waste from cattle fed the polyether ionophore antibiotics lasalocid and salinomycin. Unlike monensin, complete inhibition was not observed with these antibiotics starting at a RT of 20 days. The

TABLE 1. Comparison of data from methane-producing fermentors at 55°C which received waste from cattle fed monensin or no monensin[a]

Monensin[b]	RT (days)	CH_4 production (liters/liter per day)	Total organic acids (mg of acetate per liter)	pH	Nitrogen (mg/liter)	
					Kjeldahl	Ammonia
+	10	1.65 ± 0.03 (54)[c]	681 ± 39	7.09	1,559 ± 110[1]	538 ± 42[1]
−	10	1.78 ± 0.08 (56)	329 ± 12	7.27	1,956 ± 97[2]	772 ± 36[2]
+	6.5	2.51 ± 0.07 (52)	593 ± 32	7.00	1,895 ± 72[1]	517 ± 39[1]
−	6.5	2.62 ± 0.09 (54)	266 ± 8	7.15	2,386 ± 56[2]	652 ± 26[2]
+	Influent waste[d]		2,512 ± 157	6.38	1,693 ± 120[1]	333 ± 26
−	Influent waste[d]		2,393 ± 90	5.68	2,085 ± 89[2]	364 ± 17

[a] Data represent the mean ± standard error of two fermentors per treatment after a 6-month monensin acclimation period, with each fermentor being sampled daily for 3 consecutive days after four volume turnovers. Means within each RT or for the influent waste not having a common numerical superscript differ ($P < 0.05$).
[b] Cattle rations were supplemented with 29 mg of monensin per kg of feed.
[c] Numbers in parentheses represent the percent methane in the biogas.
[d] Mean ± the standard error of two samples.

fermentors were maintained at a 20-day RT for 2 weeks, a 10-day RT during weeks 3 through 5, and a 6.5-day RT during week 6. Methane production rates of 2.53, 2.30, ($P < 0.05$), and 2.66 liters/liter per day were obtained from the salinomycin, lasalocid, and control fermentors, respectively, under steady-state conditions at a 6.5-day RT. Under these same steady-state conditions, 0.6 mM propionate was observed in the lasalocid fermentors, while none was found in the salinomycin or control fermentors (Table 2). Similar to the fermentors fed waste containing monensin, these fermentors also had lower nitrogen levels ($P < 0.05$) and pH and somewhat lower rates of methane production and percent methane in the gas and failed when operated at a 4-day RT (Table 3). It was also determined that fermentors fed control waste at a 10-day RT could be fed waste containing lasalocid or salinomycin at the rate for the 10-day RT without significantly ($P > 0.05$) reducing methane production. However, when these lasalocid and salinomycin fermentors (10-day RT) were fed waste containing monensin at a 10-day RT, these fermentors gradually deteriorated and failed after 3 weeks.

A comparison of data from fermentors which received beef cattle waste with or without addi-

TABLE 2. Comparison of VFAs from methane-producing fermentors at 55°C which received waste from cattle fed either salinomycin, lasalocid, monensin, or no antibiotic

Time (wk)[a]	Antibiotic added[b]	RT (days)	Acid concn (mM)		
			Acetate	Propionate	Butyrate
5	Salinomycin[c]	10	1.9	0	0.1
	Lasalocid[c]	10	2.5	1.0	0
	Monensin[d]	10	4.8	3.9	0
	None[c]	10	3.9	0	0
10	Salinomycin[d]	6.5	2.3	0	0
	Lasalocid[d]	6.5	3.1	0.6	0
	Monensin[d]	6.5	5.1	2.9	0
	None[d]	6.5	2.7	0.1	0
(Influent waste)[e]	Salinomycin		26.5	5.8	8.1
	Lasalocid		21.5	7.5	5.6
	Monensin		30.6	7.0	7.5
	None		34.0	7.1	7.0

[a] Weeks after antibiotic was added, except for monensin for which footnote *a* of Table 1 applies.
[b] Cattle rations were supplemented with 29, 20, and 16.5 mg per kg of feed for monensin, lasalocid, and salinomycin, respectively.
[c] Mean of two fermentors, one sample per fermentor.
[d] Mean during steady-state conditions.
[e] Mean of two samples.

TABLE 3. Comparison of data from methane-producing fermentors at 55°C which received waste from cattle fed either salinomycin, lasalocid, or no antibiotic

Time (wk)[a]	Antibiotic added[b]	RT (days)	CH_4 production (liters/liter per day)	Total organic acids (mg of acetate per liter)	pH	Nitrogen (mg/liter)	
						Kjeldahl	Ammonia
1	Salinomycin	20	0.87 ± 0.02 (56)[c]	229 ± 25	7.35	2,106 ± 67	691 ± 51
	Lasalocid	20	1.01 ± 0.04 (57)	194 ± 19	7.35	1,976 ± 100	652 ± 59
	None	20	1.01 ± 0.04 (58)	233 ± 17	7.46	2,063 ± 89	765 ± 76
3	Salinomycin	10	1.54 ± 0.10 (53)	214 ± 7	7.28	1,890 ± 49	663 ± 80
	Lasalocid	10	1.42 ± 0.12 (52)	257 ± 19	7.26	1,753 ± 80	668 ± 59
	None	10	1.67 ± 0.03 (56)	404 ± 20	7.39		615 ± 34
5	Salinomycin	10	1.71 ± 0.07 (52)	180 ± 16	7.23	1,882 ± 32	636 ± 50
	Lasalocid	10	1.52 ± 0.03 (53)	232 ± 13	7.21	1,715 ± 78	573 ± 79
	None	10	1.81 ± 0.10 (55)	325 ± 22	7.30	2,165 ± 99	698 ± 19
10	Salinomycin	6.5	2.53 ± 0.10[1,3] (50)	243 ± 9	6.90	1,831 ± 29[1]	247 ± 41[1]
	Lasalocid	6.5	2.30 ± 0.09[2] (51)	368 ± 14	6.92	1,970 ± 46[1]	338 ± 27[1]
	None	6.5	2.66 ± 0.07[3] (54)	273 ± 6	7.16	2,418 ± 48[2]	647 ± 19[2]
(Influent waste)	Salinomycin			2,374 ± 139	6.90	1,705 ± 79[1]	358 ± 21
	Lasalocid			2,115 ± 101	6.97	1,756 ± 109[1]	328 ± 37
	None			2,393 ± 90	5.68	2,085 ± 89[2]	364 ± 17

[a] Weeks after antibiotic was added. Data from weeks 1, 3, and 5 represent the mean ± the standard error of two fermentors per treatment, one sample per fermentor; data from week 10 were obtained during steady-state conditions. Means within each RT or for the influent waste not having a common numerical superscript differ ($P < 0.05$).

[b] Cattle rations were supplemented with 20 mg of lasalocid or 16.5 mg of salinomycin per kg of feed.

[c] Numbers in parentheses represent the percent methane in the biogas.

tions of the glycoprotein antibiotic avoparcin is shown in Table 4. This antibiotic was added directly to the fermentors, whereas the previous ones were fed as dietary supplements to the animals. Avoparcin at 5 mg/liter initiated an increase in total organic acids from 599 (0 week) to 787 mg/liter (as acetate) after 1 week. The acids continued to increase gradually with increasing concentrations of avoparcin up to 15 mg/liter until week 6 when they began to decline, i.e., 1,672 to 1,077 mg/liter. Unlike the polyether ionophore antibiotics, we were able to successfully operate fermentors with 45 mg of avoparcin added per liter at a 4-day RT (data not shown).

TABLE 4. Comparison of data from methane-producing fermentors at 55°C which received cattle waste with or without avoparcin added

Time (wk)[a]	Antibiotics added (mg/liter)	RT (days)	CH_4 production (liters/liter per day)	Total organic acids (mg of acetate per liter)	pH
1	Avoparcin (5)	20	0.94 ± 0.06 (55)[b]	787 ± 37	7.78
	None	20	1.10 ± 0.04 (56)	404 ± 22	7.73
4	Avoparcin (15)	20	0.72 ± 0.09 (53)	1,672 ± 239	7.53
	None	20	0.93 ± 0.03 (56)	346 ± 19	7.66
6	Avoparcin (15)	20	0.98 ± 0.11 (54)	1,077 ± 143	7.69
	None	20	1.22 ± 0.04 (57)	548 ± 33	7.68
13	Avoparcin (40)	20	0.85 ± 0.07 (55)	354 ± 43	7.58
	None	20	1.17 ± 0.05 (58)	364 ± 33	7.64
23	Avoparcin (45)	6.5	2.46 ± 0.11 (53)	512 ± 22	7.15
	None	6.5	2.59 ± 0.13 (55)	301 ± 14	7.25

[a] Weeks after avoparcin was added. Data from weeks 1, 4, 6, and 13 represent the mean ± the standard error of two fermentors per treatment, one sample per fermentor; data from week 23 were obtained during steady-state conditions.

[b] Numbers in parentheses represent the percent methane in the biogas.

Trends observed at RTs of 6.5 and 4 days that were similar to the other antibiotics were percent methane in the gas, ammonia, and pH, which all were somewhat lower than in controls. Ammonia and Kjeldahl nitrogen were not determined every week. However, during steady-state conditions at a RT of 6.5 days, ammonia and Kjeldahl nitrogen were 629 and 2,325 mg/liter, respectively, compared with 728 and 2,533 mg/liter, respectively, for the controls ($P > 0.05$). The acetate and propionate concentrations were essentially the same for the avoparcin fermentors and controls at the 6.5-day RT, with only a trace quantity of propionate present in either of the fermentors.

Results obtained from fermentors at 35°C fed at a 20-day RT with 6% waste VSs containing the above antibiotics showed that they responded in a manner similar to the fermentors at 55°C. Inhibition of methane production was not a problem with salinomycin or lasalocid, while waste containing monensin again required an acclimation period of 6 months in the fermentors at 35°C before methane production was comparable to controls. Minimum RTs were not determined for the fermentors at 35°C.

DISCUSSION

The results of this study indicate that an extended acclimation period is necessary before microorganisms in methane-producing fermentors are able to effectively degrade waste from cattle fed the polyether ionophore antibiotic monensin. Once the microorganisms have adapted, which required 6 months under our conditions, the rate of methane production is similar to waste containing no antibiotics. In monensin-acclimated fermentors, the concentration of organic acids was doubled, the nitrogen levels including ammonia were lower ($P < 0.05$), and the pH values were lower than for control fermentors. Fuller and Johnson (8) reported depressed methane and ammonia production when monensin or lasalocid was added to a simulated rumen fermentation of high grain or roughage substrates. Slyter (15) also found decreased ammonia production during fermentation of concentrated substrates supplemented with monensin. Although the alkalinity concentrations (buffering capacity) are not shown in Table 1 for the monensin fermentors, they were observed to be approximately one-half those for the control fermentors. The lowered buffering capacity is undoubtedly a result of the lower ammonia concentration, i.e., less protein catabolism, which results in a lower pH. Whetstone et al. (21) have shown that monensin inhibits the degradation of protein by rumen microbes in vitro. This lowered buffering capacity along with increased accumulation of VFAs is presumably instrumental in the failure or washout of the microorganisms in the monensin fermentors when stressed with a 4-day RT, whereas cultures in the control fermentors were successfully maintained at this RT.

Somewhat surprisingly, waste from cattle fed two other polyether ionophore antibiotics, lasalocid and salinomycin, when initially added to fermentors at 55 and 35°C, caused no inhibitory problems. These wastes could also be added beginning with a 10-day RT and 6% VSs without inhibiting the methane production rate. This indicates that the microflora in methane-producing fermentors readily acclimates to wastes which contain lasalocid or salinomycin, yet when waste is added which contains a similar ionophore, monensin, the microbial species are inhibited. This suggests different mechanisms of action of similar polyether ionophore compounds. It is possible that the monensin inhibition is in part due to the concentration fed to the animals. Cattle rations were supplemented with 29, 20, and 16.5 mg per kg of feed for monensin, lasalocid, and salinomycin, respectively, as recommended by the various manufacturers. However, because the concentrations are not radically different, we speculate that these ionophores produce a slightly different response from one or more of the major metabolic bacterial groups active in methane fermentors (6). Monensin and lasalocid are Na^+ and K^+ ionophores (2, 13), respectively, suggesting that Na^+ transport is modified in specific microorganisms which are essential to the organic matter degradation scheme in methane fermentors., Apparently, lasalocid does not interfere with membrane transport of K^+ in critical species or groups of microorganisms such that carbon flow to methane and carbon dioxide is inhibited.

Hilpert et al. (11) have shown that growth inhibition of pure cultures of the methanogens is very pronounced when they are exposed to monensin and lasalocid. The methanogens were also shown to recover from inhibition after several days. However, others have suggested that these antibiotics do not affect the methanogens directly but inhibit the formation of methane precursors such as hydrogen and formate (7, 10, 15, 17, 18).

Avoparcin produced a short-term (6 weeks) inhibitory response when added to methane fermentors. Although it exhibited effects similar to the polyether ionophores, such as lower pH and ammonia concentrations, these fermentors did not fail at the short RT of 4 days, in contrast to the other ionophores. This suggests that this glycoprotein antibiotic again has a slightly different mechanism of action on the degradation of organic matter in methane fermentors.

The results of this study are significant when

considered in a practical sense. Today, more than 80% of cattle in feedlots are fed diets supplemented with monensin (12). Lasalocid has recently been approved for feeding to cattle by the Food and Drug Administration, which will likely increase the number of cattle being fed dietary growth-promoting antibiotics. The impact of these antibiotics on the feasibility of converting feedlot waste to methane could be critical if their inhibitory properties are not considered. This study indicates that once methane fermentor cultures are acclimated to the various antibiotics, although these cultures may have less stability at short RTs because of a lower buffering capacity, little problem should be encountered with fermenting feedlot waste thermophilically at RTs of 6.5 days or longer and VS concentrations of 6%.

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