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Death of Fecal Coliforms and *Mycobacterium paratuberculosis* during Fermentation of Corn and Feedlot Waste

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ABSTRACT

A continuous, solid-substrate fermentation of cracked corn grain mixed with the liquid phase of feedlot waste creates an acid environment (pH 4·1–4·4) hostile to many microorganisms. A fecal coliform bacterium isolated from beef cattle waste and Mycobacterium paratuberculosis *were killed rapidly after massive numbers of each were added to this fermentation. Approximately one-third of the fecal coliform population (*ca. 6×10^9 *cells per g fermenting material) were killed within 30 min of addition and 99% were destroyed within 2 h. Destruction of the mycobacterium was slower, but less than 50% of the initial* 4×10^7 *colony-forming units (*ca. *100 cells per cfu) survived for more than 4 h. Total destruction of both organisms occurred within 9 h. No cells of the introduced organisms persisted in any of the three chambers of the fermentor operated with a total retention time of 108 h, and none occurred in the output product. The process appears to be one that can eliminate enteric bacterial pathogens and mycobacteria from beef cattle waste recycled as a feedstuff.*

INTRODUCTION

A prime concern in refeeding animal wastes is the risk of spreading infectious agents that may be in the manure (McCaskey & Anthony, 1979).

* The mention of firm names or trade products does not imply that they are endorsed or recommended by the US Dept of Agriculture over other firms or similar products not mentioned.

Manures have been ensiled with animal rations, grain and hay or other biomass in static fermentations (e.g. McCaskey & Anthony, 1975; Knight *et al.*, 1977; Berger *et al.*, 1980) and fermented in a mechanically mixed solid-substrate fermentation of liquid from feedlot waste absorbed on to cracked corn (Rhodes & Orton, 1975). The latter process is a lactic acid fermentation based on the selective enhancement of indigenous lactobacilli in the manure to yield an acid product (pH 4·1–4·5) within 24 h. This lactic fermentation was extended to continuous processing at bench-scale, and after evaluation of various retention times it was concluded that the process maintained an acid environment hostile to pathogens; the method appears to have potential for continuously processing manure to a safe feedstuff (Hrubant *et al.*, 1989). The continuous process was tested further with unseparated manure in a pilot plant installed at a university feedlot. In this work, 132 kg of material were fermented daily and the product was successfully fed to cattle with savings in feed cost (Fedler *et al.*, 1984, 1985).

The subject of this report is the fate of massive additions of a fecal coliform bacterium and of *Mycobacterium paratuberculosis* added to continuously fermenting corn–feedlot waste in the bench-scale fermentor. The two microorganisms were chosen as indicators of the ability of bacterial pathogens to survive in this situation. Fecal coliforms are commonly considered indicators of all *Enterobacteriaceae*, and *M. paratuberculosis* is an overt pathogen. The dissemination of infectious mycobacteria by recycling wastes has been a specific concern cited by the Food and Drug Administration (1977). McCaskey & Shehane (1980) demonstrated the elimination, in a few days, of the rapidly growing mycobacteria, *M. phlei* and *M. smegmatis* from ensiled manure–ration mixtures. The mycobacterium studied in this paper is the etiologic agent of Johne's Disease of ruminants; it was used because it is a fastidious mycobacterium of economic importance, but is innocuous to man.

METHODS

Fermentation process

The bench-scale fermentor used in these studies and details of the process have been described by Hrubant *et al.* (1989). In essence, a three-chambered cylinder with baffles revolves at 0·5 rpm on its horizontal axis with periodic, simultaneous addition at one end of cracked corn and the liquid phase of feedlot waste (FLWL). The mixture moves through the fermentor at specific retention times. The fermentation of the corn–FLWL mixture is characterized by the spontaneous dominance of lactobacilli with yeasts

present at lesser but significant numbers. At retention times of 72–108 h, the fermented product has a pH of 4·1–4·5 and the odor characteristic of a heterolactic fermentation.

The fermentation was started by a single addition of corn (1600 g) plus FLWL (1030 ml) into the first chamber of the fermentor and incubating the mixture in the rotating fermentor for 36 h without further additions. Thereafter, corn (30 g) and FLWL (19 ml) were added simultaneously at 30-min intervals. This regime gave a total retention time of 108 h from time of introduction of the substrate until the processed product exited the three-stage fermentor (36 h chamber^{-1}). The fermentation was done in this continuous mode for at least 3 weeks before inoculation of test organisms to insure that it was stable and had the typical characteristics reported earlier (Hrubant *et al.*, 1989).

Feedlot waste liquid

Cattle waste was collected from a concrete floor of covered pens at a commercial feedlot in central Illinois. The animals were 6-month-old cattle that had been in pens for several months. Their diet was 70% ensiled corn grain and 20% cornforage silage or wastelage (particulates separated by screening manure collected from the pens); the ration also contained 10% of a protein-nutrient supplement containing monensin. Monensin is an antibiotic that increases feed efficiency of ruminants by approximately 10%, apparently by shifting the products of the rumen fermentation to higher molecular weight organic acids and less acetate and methane (Slyter, 1979).

The manure, 28% solids, pH 6·9, was diluted with tap-water to 15–20% solids and screened through a 30-mesh screen to remove the fibrous constituents. The resultant feedlot waste liquid contained 10·9% solids and was stored at 4°C until pumped into the fermentor.

Test cultures

A fecal coliform was isolated from manure of cattle fed monensin. The isolate was resistant to aureomycin and dihydrostreptomycin (both at 10 μg ml^{-1}). The resistance of the isolate to both antibiotics was increased stepwise by transferring large inocula in a unselective medium supplemented with lactose and containing the antibiotics. A stable culture resistant to both aureomycin and dihydrostreptomycin at 100 μg ml^{-1} was obtained. Ten isolates of this culture were grown in liquid media and these were pooled as the stock culture for this work. The culture simultaneously was deposited in the ARS Culture Collection as NRRL B-14103.

The *M. paratuberculosis* strain was obtained from the National Animal

Disease Center of ARS in Ames, Iowa. The culture was deposited in the ARS Culture Collection as NRRL B-4337.

Culture media

Background counts

Media used for enumeration of indigenous microorganisms in FLWL and in the fermenting corn–FLWL in the fermentor were: total count, Eugon agar (BBL, Bioquest Division of Becton, Dickenson and Co., Cockeysville, MD); lactobacilli, Rogosa SL agar (Difco Laboratories, Detroit, MI); fecal coliforms, FC agar (Difco); yeasts, Mycophil agar (BBL). For enumeration of starch hydrolyzing organisms, 0·5% corn starch was incorporated in Rogosa SL and in Mycophil media. Cycloheximide (5 mg ml^{-1}) was added to Rogosa SL or fecal coliform media when plating dilutions of 10^3 or lower. Antibiotics were added to Eugon agar, Rogosa SL agar, and FC media to determine the resistance of the intrinsic flora of the waste and that of the derived population during the fermentation. For these counts, dihydrostreptomycin sulfate, aureomycin–HCl, tetracycline–HCl, chloramphenicol (all at 10 $\mu g\, ml^{-1}$), and penicillin G sodium at 5 units ml^{-1} were used. Naturally, the Gram-negative coliforms were not tested with penicillin G.

Fecal coliform counts

The following media were used to detect and enumerate fecal coliforms: FC agar and FC agar without dyes and bile salts; FC broth and FC broth without dyes and bile salts in tubes with gas vials; and FC broth and FC broth without dyes and bile salts boiled at 2·5 × final concentration and brought to final volume by addition to autoclaved water in large test tubes with gas vials. FC broth was dispensed in 10 ml quantities into 16 × 150 mm test tubes or 25 ml in 25 mm × 150 mm test tubes. Aureomycin HCl (50 μg ml^{-1}) and dihydrostreptomycin sulfate (100 mg ml^{-1}) were added to all media to count the induced antibiotic-resistant fecal coliform NRRL B-14103 in the fermentation.

Mycobacterium counts

Two plating media were used to count *M. paratuberculosis* in samples taken from the fermentor: (1) Dorsett and Henley medium supplemented with 6 egg yolks per liter (added aseptically) and solidified with 1·5% Noble agar; 50 $\mu g\, ml^{-1}$ pimaricin (myprozine), 50 $\mu g\, ml^{-1}$ tetracycline, and 100 units ml^{-1} of benzylpenicillin sodium also were added. (2) Herrold's Egg Yolk Medium (Herrold, 1931) containing 100 $\mu g\, ml^{-1}$ amphotericin B, 50 μg ml^{-1} tetracycline, and 100 units ml^{-1} benzylpenicillin sodium. These two

media were used in spread plates and also they were dispensed as slants in screw-cap test tubes for inoculation by 10^0 and 10^1 dilutions of the sample.

The mycobacterium cells to be added to the fermentor were grown on a modified Dorsett and Henley (D&H) liquid medium (J. R. Thurston, personal communication). Its formulation is: L-asparagine, 14 g; K_2HPO_4, 1·42 g; sodium citrate $2H_2O$, 0·9 g; ferric ammonium citrate, 0·3 g; $MgSO_4 \cdot 7H_2O$, 1·5 g; glucose, 10 g; glycerol, 50 ml; $ZnSO_4 \cdot 7H_2O$, 0·0266 g; $CuSO_4 \cdot 5H_2O$, 0·0041 g; $CaCl_2$, 0·0666 g; $Co(NO_3)_2 \cdot 6H_2O$, 0·0013 g; distilled water, 1 liter.

Inoculation of fermentator

The fecal coliform NRRL B-14103 was grown as confluent growth on spread plates of FC agar without dyes or bile salts for 20 h at 28°C. A concentrated suspension of cells (OD_{540} of a 1:100 dilution = 0·9) was removed from the plates into 0·1% tryptone (Difco). *M. paratuberculosis* was grown in the modified Dorsett–Henley broth for 32 days at 37°C; 98% of the cells were acid-fast. Pellicles were collected on filter paper and the cell mass (105 g wet wt) was stirred in 100 ml 0·1% tryptone for 15 min on a magnetic stirrer. FLWL (50 g) was added with continuous stirring over an additional 5 min to bring the total volume to 250 ml.

To test survival/destruction of fecal coliforms, 17 ml of the inoculum suspension of culture NRRL B-14103 containing 10^{11} cells ml^{-1} were added through a sampling port into the fermenting corn–FLWL in the first (input) chamber of the fermentor.

In a separate trial, 245 ml of the cell suspension of *M. paratuberculosis* containing approximately 3×10^8 colony-forming units (cfu) ml^{-1} were added to the first chamber of the fermentor to give a ratio of 1 g cells per 20 g ferment (both wet wt). Cell clusters averaged 100 viable cells per cfu. FLWL was not added to the fermentor for a 6 h period beginning 90 min before *M. paratuberculosis* inoculation to compensate for the large volume of liquid added with the inoculum; corn continued to be added at 30-min intervals.

Tests for survival/destruction of these organisms were repeated and trials giving the longer survival times are reported.

Sampling and counts

Samples (5 g wet wt) of fermenting corn–FLWL were taken at intervals from each chamber and these were blended for 30 s with 20 ml of cold 0·1 M phosphate buffer (Sorenson, pH 7·0) in a Waring blender. The homogenate

was rinsed to a 50-ml volume in a graduated cylinder with sterile 0·1% tryptone.

Samples for background counts and fecal coliform determination were serially diluted in 0·1% tryptone from the initial 50-ml suspension. Counts were made by spread-plating in triplicate 0·3 ml of appropriate dilutions. Counts of lactobacilli and fecal coliforms resistant to antibiotics were determined respectively by plating onto Rogosa SL and fecal coliform media containing the antibiotics. Fecal coliforms were counted after incubation for 20 h at 44·5°C; other organisms were counted after incubation for 48–96 h at 28°C.

In addition, to detect any surviving fecal coliforms, 1- and 10-g samples of ferment were added directly into 10 and 25 ml of liquid FC medium in test tubes with and without dyes and bile salts and with and without antibiotics. An amount of 1 N KOH sufficient to neutralize the acid in the sample of fermented material (approximately 0·2 ml g^{-1}) was added to the media just before the sample was added. Tubes were incubated at 44·5°C and checked for gas production by surviving coliforms at 12-h intervals for 48 h.

The procedure for detection and enumeration of *M. paratuberculosis* was a modification of that used at the National Animal Disease Center (Merkal, 1971, 1973; Merkal & Richards, 1972; USDA, 1974). The initial 50-ml suspension of the 5-g samples of fermentated corn–FLWL were allowed to settle for 30 min. Aliquots (10 ml) of the supernatant (*c.* 40 ml total) were transferred into 16 mm × 125 mm screw-cap test tubes. Benzalkonium chloride (1 ml, 3%) was added to each tube to inhibit growth and cause dispersal and sedimentation of mycobacterial cells. The tubes were inverted several times and then allowed to stand for 16–20 h at room temperature. The sediment in each tube was equivalent to 1 g of sample of ferment. Sediments from two tubes were dispensed in 0·1 ml aliquots onto Dorsett–Henley + egg or Herrold slants for direct counts. Sediment from another tube was diluted 1:10 in water and 0·1 ml aliquots were inoculated into three tubes each of the same media. Sediment from the last of the four tubes was diluted serially in water and counts then were made by spread-plating 0·3 ml of each dilution in triplicate on both mycobacterial media. Screw caps of slant culture tubes were tightened immediately; after initial incubation of 1 week, plates were sealed in plastic bags (3 per bag) to minimize drying. Observations and counts were made weekly for 15 weeks; contaminated plates were incubated separately in sealed bags as contamination was detected. Plates or tubes were discarded when completely overgrown.

In all instances, plates used for the enumeration of organisms in this work were incubated under aerobic conditions.

RESULTS

Microbes in feedlot waste liquid

The numbers and kind of microorganisms in feedlot waste and in the liquid derived from it are essentially the same (Rhodes & Orton, 1975; Hrubant *et al.*, 1978). By comparison, the population in FLWL derived from the manure of cattle fed monensin differs somewhat from that reported above (Table 1). The total count of 3×10^{10} organisms g^{-1} was about 10% lower than the average counts previously determined on feedlot wastes sampled before monensin incorporation in cattle diets was commonplace. The coryneform organisms dominant (10–70% of the total count) in feedlot waste from animals not fed monensin represented only 20% of the population in this manure from animals fed monensin.

Conversely, lactobacilli constituted about 0·1% of the population (*c.* 10^6 organisms g^{-1}) in animals fed diets without monensin but constituted 80% of the population ($2{\cdot}6 \times 10^{10}$ organisms g^{-1}) in waste from monensin-fed animals. Further, starch hydrolysis was a characteristic common among the predominant lactics of animals not fed monensin (Nakamura, 1981); starch-hydrolyzing lactobacilli were not isolated from waste of cattle fed monensin. Antibiotic resistance was common among the lactobacilli in the waste used in this study, but no correlation with the use of monensin is inferred.

TABLE 1
Aerobic Microorganisms (number of organisms g^{-1} wet wt) in Feedlot Waste Liquid[a] from Monensin-Fed Cattle

Group	*Total*	*Antibiotic resistance to*				
		Strep[b]	*Terra*	*Aureo*	*Pen G*	*Chloram*
Total aerobes	$3{\cdot}3 \times 10^9$	1×10^8[c]	0	0	2×10^6	3×10^5
dominant coryneform	$7{\cdot}3 \times 10^8$	0	0	0	0	0
Lactobacilli	$2{\cdot}8 \times 10^9$	$2{\cdot}6 \times 10^9$	$7{\cdot}0 \times 10^8$	$2{\cdot}8 \times 10^8$	$3{\cdot}3 \times 10^7$	$8{\cdot}3 \times 10^4$
Fecal coliforms	$1{\cdot}7 \times 10^6$	$1{\cdot}4 \times 10^5$	$3{\cdot}4 \times 10^5$	$4{\cdot}1 \times 10^5$	—[d]	<10
Yeast	$2{\cdot}0 \times 10^5$	—	—	—	—	—

[a] FLWL: 10·9% dry matter; pH 6·9.
[b] Antibiotics: Strep = dihydrostreptomycin sulfate, 10 $\mu g\,ml^{-1}$; terra = Terramycin–HCl, 10 $\mu g\,ml^{-1}$; aureo = aureomycin–HCl, 10 $\mu g\,ml^{-1}$; pen G = benzylpenicillin sodium, 5 units ml^{-1}; chloram = chloramphenicol, 10 $\mu g\,ml^{-1}$.
[c] Lactobacilli ('pinpoint' colonies) excluded from total aerobes showing antibiotic resistance.
[d] Analysis not done.

The number of fecal coliforms, $1{\cdot}5 \times 10^7\ g^{-1}$, in the FLWL of monensin-fed cattle was 10- to 100-fold lower than in the FLWL from animals fed diets without monensin. Between 10 and 20% of the fecal coliforms were resistant to streptomycin or the tetracyclines; however, only 0·2% were resistant to the combination of streptomycin and aureomycin at the concentrations used to assess destruction of the resistant fecal coliform isolate inoculated into the fermentation.

The number of yeasts (*c.* $10^6\ g^{-1}$) was about the same in this FLWL from cattle fed monensin as was found in earlier work.

Corn and FLWL fermentation

Changes in the microbial population of the corn–FLWL mixture during the initial 36-h period of batch operation are shown in Fig. 1. The overall pattern is typical of this fermentation in batch mode (Rhodes & Orton, 1975). The

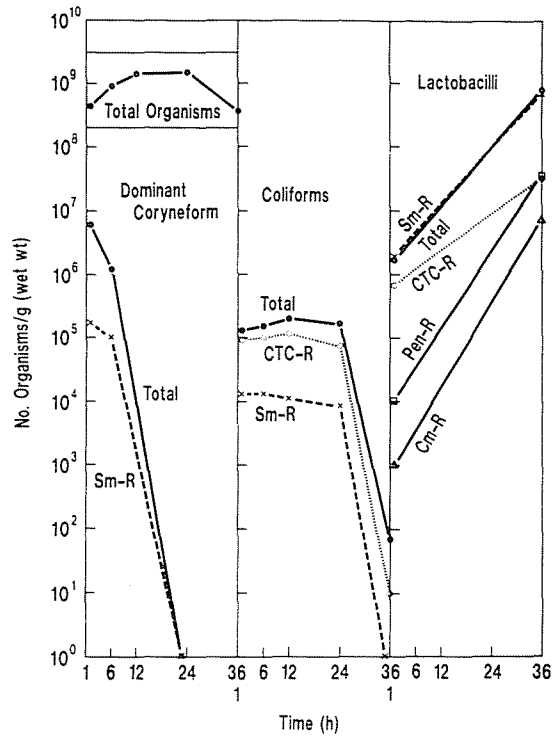

Fig. 1. Changes in microbial population of corn–FLWL mixture during initiation of fermentation in three-chambered fermentor, Antibiotic-resistant populations indicated by: Sm-R, resistant to $10\,\mu g\,ml^{-1}$ dihydrostreptomycin; CTC-R, resistant to $10\,\mu g\,ml^{-1}$ chlortetracycline (aureomycin); Pen-R, resistant to 5 units ml^{-1} benzylpenicillin G; and Cm, resistant to $10\,\mu g\,ml^{-1}$ chloramphenicol. Dominant coryneforms resistant to CTC, Cm, or Pen and coliforms resistant to Cm were not present in the population.

total count characterized by the dominant numbers of coryneform organisms decreased to negligible numbers within 24 h and there was a reduction in numbers of total coliforms by the end of 36 h. Concomitantly, the population of lactobacilli substantially increased, although the numbers did not match those obtained with FLWL from animals not fed monensin. There was no marked selectivity associated with antibiotic resistance during changes in the flora, although there was retarded increase in the numbers of aureomycin-resistant lactobacilli (CTC-R) compared to other lactobacilli.

Subsequent operation in a continuous mode maintained the characteristics evident during batch operation: a dominant population of lactobacilli (10^{10} g^{-1}) with a significant number of yeasts (10^7 g^{-1}). When detected, fecal coliforms numbered about 10^1 in the first chamber, but were never present in the others. As reported earlier, lactobacilli capable of hydrolyzing starch appeared during the continuous operation. Although these numbers are about one-tenth those reported for fermentations using FLWL from the manure of cattle not fed monensin, the pH of the fermented material averaged 4·45, 4·35 and 4·1 in chambers 1 (input), 2 and 3, respectively. Moisture content was 45% in the first chamber and slightly higher in the other two.

Destruction of fecal coliforms

Preliminary tests showed that the same plate counts of the test fecal coliform, NRRL B-14103, were obtained on FC agar and FC agar without dyes and bile salts whether or not aureomycin (50 $\mu g\,ml^{-1}$) and dihydrostreptomycin (100 $\mu g\,ml^{-1}$) were incorporated. Gas production occurred with both 1- and 10-g samples in liquid FC and liquid FC without dyes or bile salts as well as when antibiotics were included.

The distribution of the test fecal coliform in the fermentor after addition of $1{\cdot}7 \times 10^{12}$ cells into the approximately 2600 g (wet wt) of FLWL + corn in the first chamber is shown in Fig. 2. By the end of the first hour, samples from chamber 1 yielded both large and small colonies on FC agar whereas colonies on FC agar without dyes or bile salts were uniform. Thus, a significant number of fecal coliform cells were damaged by short exposure to the acid conditions (pH 4·4) of the ferment. By 4 h after inoculation, plate counts could be made only on the non-restrictive FC medium (no colonies appeared on standard FC agar). The numbers of normal and damaged + normal cells based on the differentiation afforded by the two variants of FC agar are plotted in Fig. 2.

Small numbers of fecal coliforms, about 0·03% of the inoculum, migrated into chamber 2 within 30 min after addition. However, no fecal coliforms were detected in 1-g samples from chamber 2 beyond 2 h after inoculation

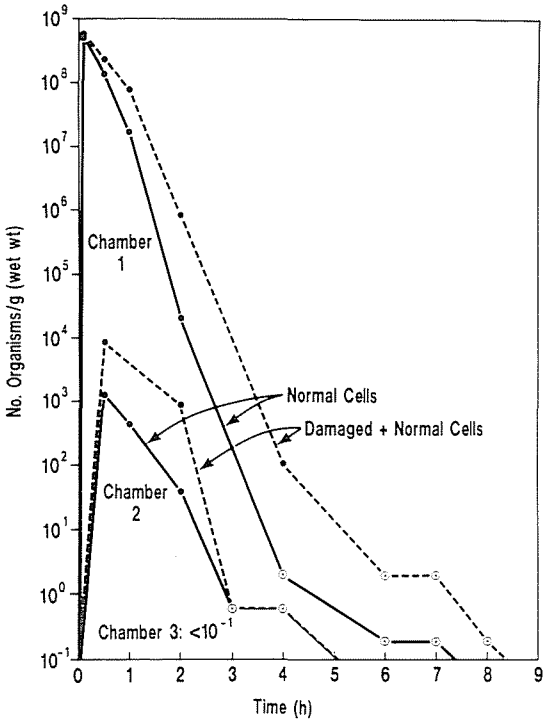

Fig. 2. Fecal coliform kill in continuous solid-substrate fermentation of corn and feedlot waste liquid at pH 4·4. Normal cells of the test strain are those able to grow on FC agar with or without added antibiotics. Damaged cells are those unable to grow on FC agar with antibiotics but able to grow on FC agar minus dyes and bile salts with antibiotics added.

and the first 10-g samples tested, 6 h after inoculation, were negative. Viable fecal coliforms were never detected in chamber 3.

Overall, one-third of the fecal coliforms were killed within 30 min after addition to the fermentor and 27% of the remaining cells were injured, as measured by differential counts on restrictive versus non-restrictive FC media. After 2 h incubation, 99·99% of fecal coliforms were dead and 95% of the remainder were injured. By 4 h, only damaged cells persisted in the fermentor and these disappeared thereafter.

Destruction of *M. paratuberculosis*

Following inoculation, approximately 5% of the wet weight of the fermenting mixture in chamber 1 was *M. paratuberculosis* cells. More than 98% of the cells were acid-fast. The method of inoculum preparation retained clumped cells such that the cfu averaged 100 cells per unit. These

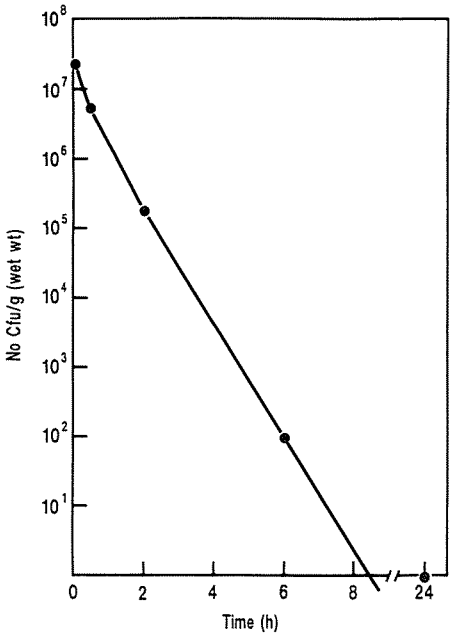

Fig. 3. *Mycobacterium paratuberculosis* kill in continuous fermentor. Colony-forming units (cfu) averaged 100 organisms per unit. All cells were killed in the first chamber.

aggregates simulated the state of mycobacteria shed in excreta of infected cattle (Merkal, 1971, 1973).

As shown in Fig. 3, *M. paratuberculosis* was killed within 9 h after introduction in chamber 1 of the fermentor. *M. paratuberculosis* was not detected in samples from chamber 2, chamber 3, or the output material over a 14-day test period. The FLWL itself was not inhibitory to growth of this mycobacterium; in a sterile control medium consisting of 1 part FLWL with 3 parts H_2O, counts of *M. paratuberculosis* doubled in 10 days.

Data shown in Fig. 3 are the highest viable count obtained on either Dorsett–Henley + egg or Herrold media on either slants or plates. Microbial counts were essentially the same on both media. Incubation of slants was terminated at 15 weeks, by which time contamination precluded further retention. Colonies on plates were counted first after 4 weeks; by 6 weeks, growth was confluent on plates containing more then 60 cfu. Colony counts did not increase during the interval between 6 weeks and up to 15 weeks when incubation was terminated.

DISCUSSION

Both the fecal coliform and *M. paratuberculosis* were killed rapidly in the acid ferment of the solid-substrate fermentation of corn plus feedlot waste liquid.

Death of fecal coliforms in this heterolactic fermentation corresponds to reports of the lethal effect of lactic and acetic acids (Roth & Keenan, 1971) and of sodium acetate buffers on fecal coliforms (Przybylski & Witter, 1979). Damaged cells persisted 1 h after normal cells no longer could be detected; the use of resuscitation media or buffers containing K_2HPO_4 might have eliminated this differential (Mossel & Ratto, 1970). This study indicates that fecal coliforms do not survive in this fermentation milieu for more than a few hours.

The time required to kill the mycobacterium was about the same as that for fecal coliforms, even though the mycobacterial cells were in large clumps. In addition to death from hydrogen ion concentration, it is possible that the waxy coat of the *M. paratuberculosis* cells absorbed the undissociated acids with lethal results.

The results of these studies on the death of test organisms show that: (1) the inclusion of monensin in cattle rations does not significantly alter the lactic acid fermentation of corn mixed with the liquid derived from the manure of those cattle; (2) fecal coliforms and mycobacteria are killed within 9 h of exposure to the fermentation conditions of this process even though the one-time test inoculum introduced at least 10 000 times the number of cells that might be expected in the waste under normal conditions (fecal coliforms) or from infected animals (mycobacteria); (3) the design of the fermentor ensured mixing of the flora and solid substrate such that cells of the test organisms did not migrate through the fermentor unaffected by the acid conditions of the fermentation; and (4) pathogens, as exemplified by the test organisms, will not appear in the fermented product at retention times of 72–108 h and thus the solid-substrate process is a safe method for recycling animal wastes as a feedstuff.

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