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Swine Waste-Corn Silage and the Survival of Dysentery Bacteria

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ABSTRACT

SILAGE fermentation of swine waste combined with corn undergoes a lactic acid fermentation which quickly kills fecal coliform bacteria, *Salmonella derby* and dysenteric *Treponema hyodysenteriae* organisms. The product of this fermentation process may be used as a major component of animal rations and as an alternative method of animal waste management.

INTRODUCTION

Swine waste is produced in the United States at the rate of 146 million metric tons per year (Weiner, 1980), and management of this material continues to cause problems in total confinement production systems. Additional methods of handling swine waste other than storage and application to land would be especially valuable if the process recovers nutrients still present in waste and reduces environmental pollution (Kornegay et al., 1977). Refeeding waste as part of an animal ration represents one such additional method, but the procedure has a disease potential (Bhattacharya and Taylor, 1975; Fontenot and Webb, 1975) that can be diminished by fermentation processes (McCaskey and Anthony, 1979; Creger et al., 1973). Swine waste combined with cracked corn in an anaerobic vessel results in a lactic acid fermentation with pH levels near 4 (Weiner, 1982). This investigation examined the effect of such a lactic acid fermentation on the survival of fecal coliform microorganisms and of the pathogenic swine dysentery bacteria, *Salmonella* sp. serotype *derby* NRRL 14064 ("S. derby") and *Treponema hyodysenteriae* B204.

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MATERIALS AND METHODS

Fresh swine waste was obtained from a feedlot in Central Illinois which finished hogs in an open-front barn of partially paved and dirt surfaces. Waste (1.2 kg) was mixed with distilled water to give a final moisture content in silage of 40% (Weiner, 1982). Either fecal coliform bacteria, "*S. derby*" or *T. hyodysenteriae* was added to the waste and mixed before combining with cracked corn (2.5 kg). A laboratory silo with a newly designed sampling port was filled from the top with this mixture with intermittent tamping. The silo was closed as described below and placed in an incubator at 30 °C (Weiner, 1980).

The Laboratory Silo

A 14.2 × 25 cm cylinder made of 0.64 cm thick transparent acrylic plastic (Leucite) which provided a capacity of 4 L was used to ensile the swine waste-corn mixture (Fig. 1). Leucite lids, 2.5 cm thick, were fitted into the cylinder to a 1.0 cm depth by step construction and pressed against rubber "O" rings to ensure airtightness. The lids were held in place by tightening thumb screws on each of four 0.64 cm threaded steel rods that passed through holes in the lids. The vessel was

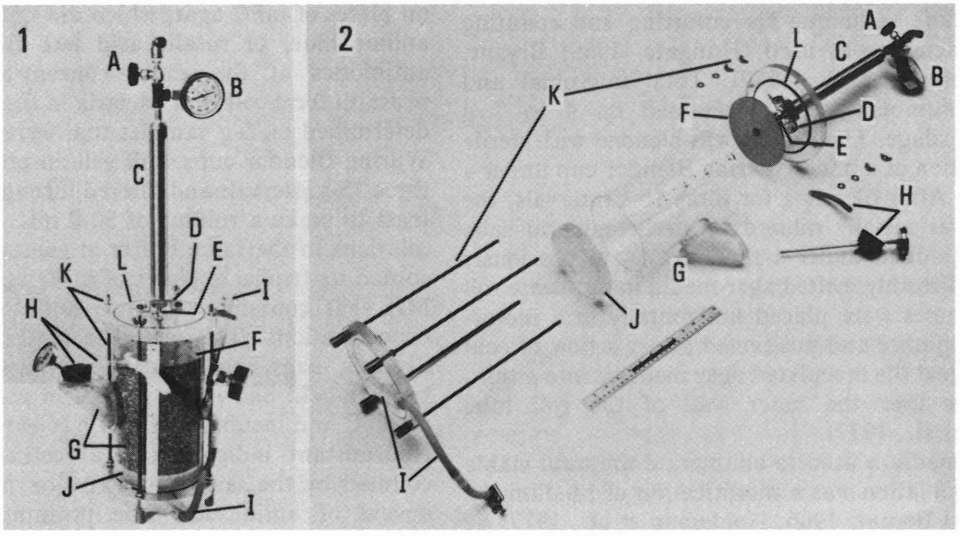

Fig. 1—Laboratory silo with fermenting swine waste-corn (1), and exploded view (2) of components. A, Hoke valve; B, gas gauge for air cylinder; C, air cylinder; D, platform, bolted to lid of silo to hold air cylinder; E, piston from air cylinder; F, plate pressed down on silage surface by piston; G, sampling port and fermentor cylinder; H, rubber stopper containing a dial thermometer and Bunsen valve; I, horizontal channel and hose outlet for release of silage liquid; J, sieve disc to separate out silage liquid; K, threaded steel rods and thumb screws to hold fermentation cylinder and lids in place; L, top "O" ring, set in step construction of inside of lid.

fitted centrally with a single Leucite sampling port constructed in three stages. The first construction was a short cylinder formed to glue (1,2-dichloroethane) outside around a central hole in the silo wall. Secondly, a flat plate with a round hole was glued to the short cylinder. Next, a tube was fitted to the plate at a 45 deg angle. A second plate, taper drilled to accommodate a large rubber stopper, was glued to the top end of the 45 deg tube to provide an entrance orifice parallel to a bench top. During sampling, the port was flushed with oxygen-free, sterile carbon dioxide by way of a bent sterile needle. Carbon dioxide flowed out over the sides of the port and maintained an anaerobic blanket over the opening as the sample was transferred to a similarly flushed sample jar.

Silage liquid exited from the bottom through a perforated Leucite disc resting on the bottom lid. The disc was hollowed on its lower surface, as was the upper portion of the bottom lid, to form a lens-shaped cavity. A center hole in the bottom lid was connected to a horizontal channel drilled through a glued-on solid rectangle of plastic. The horizontal channel terminated in a tube shape, and an attached rubber tubing with clamp served as a valve for release of silage liquid.

Continuous silage compaction to simulate cylindrical farm silos (El Hag et al., 1982; Otis et al., 1959; Otis, 1950) was achieved by a pneumatically driven piston-cylinder system attached to a platform held on the silo lid. The stainless steel piston of the air cylinder passed through a gland in the lid fitted with a rubber "O" ring, and the piston fastened into a stainless-steel plate that pressed downward on the silage surface. The downward pressure was effected by passing compressed nitrogen through a Hoke valve and a gas pressure gauge to the air cylinder; a pressure of 14.1 kg/cm^2 (200 psi) was used. The rubber stopper in the sampling port was drilled to accommodate a Bunsen valve, which consisted of rubber tubing with a sealed end and a small slit, through which internal gas pressure could be released but entrance of outer air would be prevented.

Microbiology

The roll tube technique for culturing and counting anaerobic bacteria was used (Hungate, 1969; Bryant, 1972; Holdeman et al., 1977). Total microbial and lactobacilli numbers were determined on 4 to 7 g quantities of silage. The sample was blended with sterile mineral solution in a micro Waring Blendor cup under a flow of CO_2. After blending for three 15-s intervals, the top liquid was serially diluted in sterile mineral salts solution and added under a flow of CO_2 to previously prepared and freshly melted agar media in duplicate roll tubes. The tubes were placed horizontally in a motor-driven tube spinner and positioned under a flow of cold water to congeal the inoculated agar medium into a thin, smooth layer over the inner wall of the roll tube (Holdeman et al., 1977).

The agar medium used to enumerate the total viable microbial population was a modification of Medium 10 (Caldwell and Bryant, 1966; Holdeman et al., 1977) by using glucose and cellobiose at half concentrations of 0.025%. This medium was prepared by boiling in round-bottomed flasks under a flow of CO_2. A rubber stopper was wired in place and the flask was autoclaved 15 min at 121 °C. The procedure that pre-reduced and anaerobically sterilized the medium was designated PRAS. After sterilization of the medium and cooling to 47 °C, PRAS solutions of 8% Na_2CO_3 (5 mL) and 2.5% cysteine $HCl{\cdot}H_2O$ (2 mL) were added to the flask. Medium was dispensed at the rate of 9 mL per sterile roll tube, into which a rubber stopper was tightly inserted.

Lactobacilli were enumerated on Rogosa SL Agar (RSL; Difco), which was modified for use in anaerobic roll tubes as follows: To 92 mL of water were added RSL agar, 7 g; agar, 0.5 g; resazurin, 0.1%, 0.1 mL; and glacial acetic acid, 0.13 mL. After heating to boiling under CO_2, the following were added: 5 mL 8% Na_2CO_3, 2 mL cysteine·$HCl{\cdot}H_2O$ and concentrated HCl to bring the pH to 5.4. This was not autoclaved; it was directly dispensed under flowing CO_2 in 9 mL, volumes into sterile tubes, which were sealed with rubber stoppers.

Serial dilutions of the silage for enumeration of microbes were made with mineral salts solutions prepared as follows: To 93 mL of water were added mineral solution 1, 3.8 mL; mineral solution 2, 3.8 mL; and resazurin, 0.1%, 0.1 mL. The solution was pre-reduced and anaerobically sterilized at 121 °C for 15 min. The cooled solution was amended with PRAS solutions of 5 mL 8% Na_2CO_3 and 2 mL of 2.5% cysteine·$HCl{\cdot}H_2O$. Serial dilutions for determining fecal coliforms and "*S. derby*" by agar plate methods were done in 0.08 M phosphate buffer with 0.2% gelatin at pH 6.5 (Bissonnette et al., 1975). Dilutions for counting *T. hyodysenteriae* were done with 0.01 M phosphate buffer in 0.85% NaCl (PBS) at pH 7.3 (Kinyon and Harris, 1974).

The first experiment tested the survival of large numbers of added fecal coliform bacteria, which were distinguished from normal coliform flora by resistance to a combination of chlortetracycline and dihydrostreptomycin at respective concentrations of 50 and 100 μg/mL. The organism, NRRL B-14103, was kindly supplied by G. R. Hrubant from the Agricultural Research Service Culture Collection, Northern Regional Research Center, Peoria, Illinois. Inoculum for the fermentation consisted of gelatin-phosphate buffer washes (40 mL) of B-14103 cells grown for 16 h at 30 °C on plates of mFC agar, which did not contain bile salts, aniline blue, or rosalic acid but did contain the two antibiotics at the above concentrations. Antibiotic-resistant fecal coliform bacteria in the fermentation were determined on 5-g samples that were blended in sterile Waring Blendor cups with gelatin-phosphate buffer for three 15-s intervals and filtered through sterile glass wool mats to make a volume of 50.0 mL. Appropriate serial dilutions in the same buffer at volumes of 0.3 mL were spread on triplicate plates of mFC agar (Difco, Detroit, MI) that contained the two antibiotics at the above concentrations. The triplicate plates were enclosed in Zip-Lok bags, agar face down, and placed in wire baskets held below the surface of water maintained at 44.5 °C and incubated for 24 h (Geldreich et al., 1964). Nonresistant indigenous fecal coliform bacteria were counted by the same procedure on mFC agar medium devoid of antibiotics. The presumptive test for the presence of coliform bacteria was done on each silo sample by adding 1 g of sample to each of five tubes of lactose broth (APHA, 1971); gas production constituted a positive test.

The second experiment tested for the survival of "*S. derby*" in the ensiled swine waste-corn mixture. This

bacterium was grown in 100 mL dulcitol broth (Kaper et al., 1977) at 41.5 °C for 18 h and then was added to fresh swine waste diluted with distilled water. Salmonellae were enumerated by blending 5.0-g samples from the silo with sterile gelatin-phosphate buffer, and the appropriate serial dilutions were added to plates of Brilliant Green Agar with Sulphadiazine (BGS) (BBL, Division of Bioquest, Cockeysville, MD). Plates were incubated for 48 h at 41.5 °C. At the same time, Salmonellae were detected and isolated by transferring 10 g samples from the silo to 100 mL dulcitol broth and incubating 4 h at 25 °C and 20 h at 35 °C. The culture (10 mL) was transferred to 90 mL Tetrathionate Broth Base (BBL) to which Brilliant Green dye (12.5 mg/L) had been added (TETBG). The culture was incubated at 35 °C for 24 h and then streaked on plates of BGS. If typical pink-red colonies typical of salmonella did not develop, BGS plates were again streaked from the same TETBG flasks at 48 h. If presumptive salmonella colonies appeared, three typical colonies were inoculated into both Triple Sugar Iron Agar (BBL) and Lysine Iron Agar (BBL) slants which were slants incubated for 24 h at 35 °C to detect biochemical reactions typical of "*S. derby.*" The reliability of the salmonella detection procedure was tested at each sampling time by taking a pure culture of "*S. derby*" through the analytical protocol used to detect salmonella in the swine waste-corn samples.

The third experiment evaluated the survival of *Treponema hyodysenteriae* B204 in the swine waste-corn silage. This swine pathogen was kindly provided by J. M. Kinyon, Iowa State University, Ames, Iowa. The organism was grown in Trypticase Soy Broth without Dextrose (TSB, from BBL). The medium was pre-reduced and anaerobically sterilized (Kinyon and Harris, 1974) in anaerobic tubes and round-bottomed flasks, the latter fitted with rubber stoppers that had glass tubing access ports with rubber tubing (Harris, 1980). Fetal calf serum (10% for tubes and 3% for flasks) and 10% inoculum were added under a flow of 50% H_2 and 50% CO_2 (oxygen free), the flask was sealed, and the organisms were grown for 48 h at 37 °C. Successive transfers of 10% inocula from tubes to flasks provided 600 mL inoculum for the start of the silage fermentation. *Treponema hyodysenteriae* was enumerated in a selective agar medium of TSB containing 5% bovine blood and Spectinomycin, 400 mg/mL (Upjohn Company, Kalamazoo, MI) (Songer et al., 1976). A 5-g sample from the silo was blended with phosphate buffered saline (PBS), filtered through sterile glass wool pads to give 50.0 mL of filtrate, and appropriate dilutions were plated. The plates were incubated at 40 °C in an atmosphere of 80% H_2 and 20% CO_2 contained in vented Gas Pak jars (BBL) with cold palladium catalyst (Songer et al., 1976). Colonies which were beta hemolytic and were typical spirochaetes by phase microscopy were considered to be the pathogen. The protocol for detection and isolation of *T. hyodysenteriae* was monitored by inoculation of phosphate buffered saline with this organism, which was blended and filtered as was done with samples from the silo. The filtrate was streaked on duplicate TSB plates that also contained blood and Spectinomycin. The plates were incubated in the same Gas-Pak jars as the silo samples.

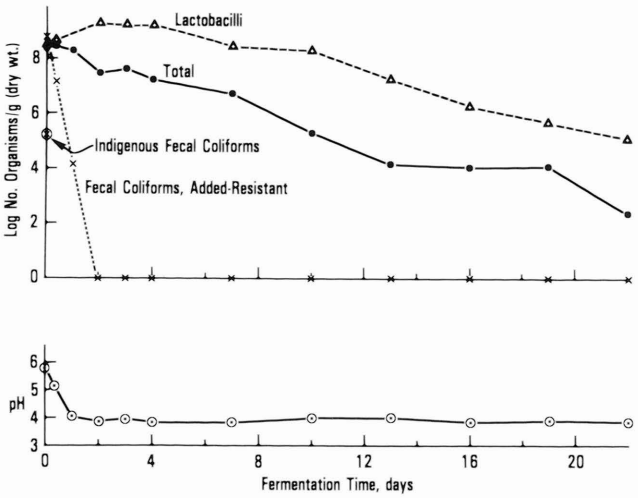

Fig. 2—Survival of antibiotic resistant fecal coliform bacteria and growth of total organisms and lactobacilli in silage fermentation of swine waste-corn.

RESULTS

Fecal Coliform Removal

Swine waste combined with corn contained (all data per dry g) 1.9 × 10^5 fecal coliform bacteria. Of this indigenous number, 8.36% were resistant to chlortetracycline and dihydrostreptomycin. At the start of the fermentation, antibiotic-resistant fecal coliform organisms were added to the swine waste-corn fermentation to give a count of 7.86 × 10^8 (Fig. 2). Resistant and normal coliform bacteria decreased rapidly to 1.31 × 10^4 at 1 day but were not detected again in subsequent fermentation samples, either as colonies in plates of selective medium or as gas producers in lactose broth. A competing population of lactobacilli with starting numbers of 3.79 × 10^8 multiplied to 3.13 × 10^9 organisms at 2 days. The total numbers of viable aerobic microorganisms were always less than the lactobacilli count. Rapid production of acid reduced the pH from initial levels of 5.8 to near 4 in 1 day and maintained this level for the duration of the fermentation.

Salmonella Destruction

Swine waste, shown to be free from salmonella, was

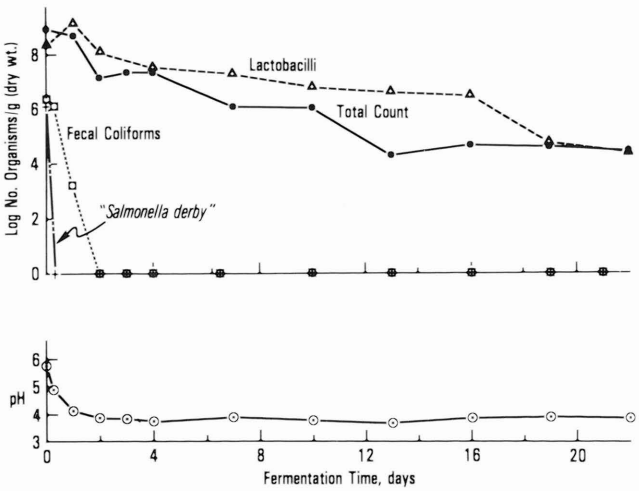

Fig. 3—Survival of *Salmonella derby* and growth of total bacteria and lactobacilli in silage of swine waste-corn fermentation.

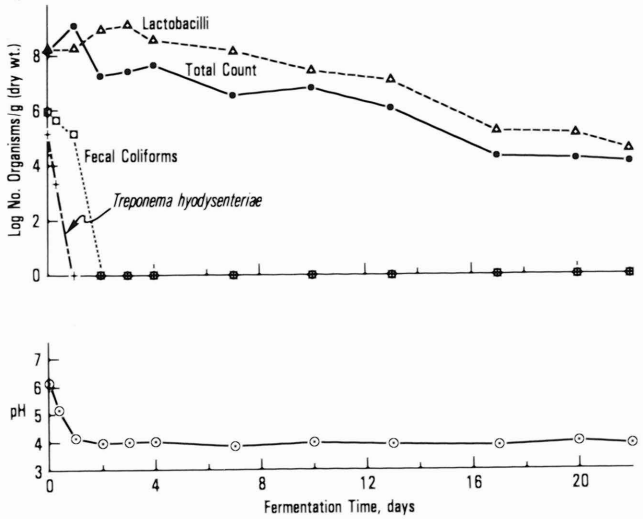

Fig. 4—Survival of dysenteric *Treponema Hyodysenteriae* and growth of total bacteria and lactobacilli in silage of swine waste-corn.

inoculated with a culture of "*S. derby*" combined with cracked corn in a silo to give an initial count of 1.14×10^6 (Fig. 3). These organisms were not detected at 8 h and were never found in 10 subsequent samples taken during the 3-wk fermentations. Positive control cultures with "*S. derby*", tested simultaneously with each fermentation sample, always showed the presence of the organism.

Indigenous fecal coliform bacteria initially numbered 3.98×10^6, decreased to 2.28×10^3 at 1 day, and were never detected thereafter either on selective medium or in lactose broth (Fig. 3). Lactobacilli initially numbered 3.67×10^8 organisms and they increased fourfold at 1 day to represent more than 99% of the total number of bacteria. This level remained for the duration of the fermentation. Acid produced by the lactobacilli reduced the pH from 5.75 to 4.10 in 1 day and for the rest of the fermentation, pH ranged from 3.69 to 3.88.

Treponema hyodysenteriae Kill

In this experiment, the number of *T. hyodysenteriae* added to swine waste combined with corn was 1.8×10^5 organisms (Fig. 4). This number decreased to 3.84×10^3 at 8 h and was never detected again in the 10 subsequent samples taken during the course of the fermentation. Test plates for counting and revealing treponemes were always accompanied by two control plates streaked with *T. hyodysenteriae.* These duplicate control plates always showed colonies surrounded by beta hemolytic zones, and the colonies were confirmed to be typical spirochaetes.

DISCUSSION

The fermentation conditions in the silo, i.e., anaerobiosis, lower moisture content than conventional ensilage operations, and a solid substrate of cracked corn coated with swine waste, encourage a selective proliferation of lactobacilli from the intrinsic flora of fresh swine waste. As aciduric lactobacilli multiply, they excrete acids, primarily lactic and a little acetic, into the common environment (Weiner, 1982). The lactic acid bacteria also produce antibacterial substances that inhibit other groups of microorganisms (Weiner, 1980; Hurst, 1973; Sandine, 1972). It is probable that acids and antibacterial compounds are toxic to other microbial groups in swine waste, particularly enteric bacteria. Thus, indigenous coliform populations were eliminated after 1 day of fermentation. The deliberate addition of coliform bacteria, labeled by resistance to the two antibiotics, was done in numbers that were over 4000-fold greater than normal populations. Even these large numbers were killed after 1 day of fermentation. Fecal coliform bacteria not only are utilized as indicator organisms of fecal pollution, but certain species such as enteropathogenic *Escherichia coli* also cause dysentery.

An enteric pathogen that was added to a silo fermentation of swine waste-corn was *Salmonella* sp. serotype *derby.* It was chosen as an example of a salmonella infectious agent because this bacterium was the most common isolate (37.5%) from fecal samples of swine at slaughter plants in six states representing 64% of United States hog production (McKinley et al., 1980). "*Salmonella derby*" organisms were more sensitive to destruction than fecal coliform bacteria in anaerobic lactic acid fermentations. The procedure for the detection of salmonella included resuscitative measures for organisms damaged by environmental stresses (Bissonnette et al., 1975). For example, lower incubator temperatures than the selective level of 41.5 °C may be required for salmonella detection with samples containing small populations of these bacteria (Harvey and Price, 1968). Also, it has been shown that stressed fecal indicator coliforms may be revived by use of nonselective media (Kaper et al., 1977). Thus, the salmonella detection protocol included initial culture in dulcitol broth at 25 °C. Even with the application of resuscitative measures, "*S. derby*" could not be detected in the fermentation samples.

Treponema hyodysenteria has been recognized as the major cause of swine dysentery (Kinyon and Harris, 1974) and represents an important pathogen that is transmitted by fecal contamination. Thus, it was important to learn through experiments done with swine waste inoculated with this pathogen that the ensiling process has the capacity to control transmission of the disease. Importantly, the lactic acid fermentation evidently also can kill added viruses in edible wastes, among which were a porcine strain and bovine diarrhea strain, the latter of which is similar to the hog cholera virus (Gilbert et al., 1982). It is probable that other microorganisms, including other pathogenic bacteria, would also be excluded by this microbial process because of the highly competitive character of lactic acid bacteria and the selective nature of the silage fermentation.

SUMMARY

Raw swine waste combined with corn and fermented under anaerobic conditions undergoes a lactic acid fermentation that quickly kills large reservoirs of fecal coliform bacteria, added "*S. derby*" populations, and added dysenteric *T. hyodysenteriae* organisms. The product of this fermentation process may be used as a major component of animal rations and as an alternative method for the management of animal waste.

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