

Fate of *Escherichia coli* O157:H7 in Manure-Amended Soil

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***Escherichia coli* O157:H7 cells survived for up to 77, >226, and 231 days in manure-amended autoclaved soil held at 5, 15, and 21°C, respectively. Pathogen populations declined more rapidly in manure-amended unautoclaved soil under the same conditions, likely due to antagonistic interactions with indigenous soil microorganisms. *E. coli* O157:H7 cells were inactivated more rapidly in both autoclaved and unautoclaved soils amended with manure at a ratio of 1 part manure to 10 parts soil at 15 and 21°C than in soil samples containing dilute amounts of manure. The manure-to-soil ratio, soil temperature, and indigenous microorganisms of the soil appear to be contributory factors to the pathogen's survival in manure-amended soil.**

Animal waste in the form of raw manure or composted manure is routinely applied to the land as a crop fertilizer and/or soil amendment. Compost also is used as a growth medium in home gardening, ornamental nurseries, and greenhouses (9). A potential risk arising from the disposal of animal waste of fecal origin is the spread of enteric pathogens (15). Animals from which food is derived are recognized as reservoirs of many significant food-borne pathogens, including *Escherichia coli* O157:H7, *Salmonella* spp., and *Campylobacter* spp. (6, 7). Many outbreaks or cases of *E. coli* O157:H7 infection have been associated with water or food directly or indirectly contaminated with animal manure (1–4). For example, an outbreak of *E. coli* O157:H7 infection among members of four families was associated with food fertilized with cattle manure on the farm (3). In another instance, a woman acquired *E. coli* O157:H7 infection associated with eating inadequately washed vegetables that were obtained from a garden fertilized with bovine manure (4).

Previous studies on the fate of *E. coli* O157:H7 in bovine feces revealed that the pathogen survived for 42 to 49 days at 37°C, for 49 to 56 days at 22°C, and for 63 to 70 days at 5°C (17). Another study of *E. coli* O157:H7 in manure heaps revealed that the pathogen could survive for up to 47 days, 4 months, and 21 months in bovine, aerated ovine, and nonaerated ovine manure, respectively (12). Himathongkham et al. (11), based on their research findings, recommended that cow manure should be held for 105 days at 4°C or 45 days at 37°C to achieve a 5- \log_{10} reduction of both *E. coli* O157:H7 and *Salmonella enterica* serovar Typhimurium. These results indicate that *E. coli* O157:H7 can persist in bovine feces for an extended period of time and that bovine feces are a potential vehicle for transmitting the pathogen to cattle, food, and the environment.

Cross-contamination of produce from manure or improperly composted manure used as a soil amendment may be a source of pathogen contamination during preharvest. Although com-

petition with soil microorganisms and adverse environmental conditions can influence pathogen survival, there is little information regarding the degree to which *E. coli* O157:H7 cells can survive in manure-amended soils. In this study, our objective was to determine the fate of *E. coli* O157:H7 cells in cow manure applied to soil at different manure application rates and held at different temperatures.

A five-strain mixture of *E. coli* O157:H7 cells (strains E0143 [meat isolate], C7927 [human isolate], K262 [human isolate], C0083 [cattle feces isolate], and E0139 [beef jerky isolate]) was used as the inoculum. In order to facilitate enumeration of these isolates, all five strains of *E. coli* O157:H7 were labeled with jellyfish green fluorescent protein (GFP) according to the protocol described by Delazari et al. (5), with slight modification. The competent bacterial cells were electroporated in a Gene Pulser II (Bio-Rad) with plasmid vector pGFPuv (Clontech, Palo Alto, Calif.), and an electrical pulse ($\tau \sim 1.7$ msec) was applied at 2.5 kV and 25 μ F with the pulse controller adjusted to 200 Ω . Transformants were selected from isolated colonies grown on Luria-Bertani (LB) plates containing 100 μ g of ampicillin/ml. The resulting ampicillin-resistant transformants emitted bright green fluorescence under a handheld UV light.

Each strain of GFP-labeled *E. coli* O157:H7 was inoculated into 10 ml of Tryptic soy broth (TSB; Becton Dickinson, Sparks, Md.) containing 100 μ g ampicillin/ml (TSB-A) and was incubated at 37°C for 16 to 18 h with agitation (150 rpm). A 0.5-ml suspension of each isolate was transferred to 100 ml of TSB containing ampicillin (100 μ g/ml) and incubated for 16 to 18 h with agitation (150 rpm). The bacteria were harvested by centrifugation (4,000 $\times g$ for 20 min) and were washed three times in 0.1% peptone water. Each strain was adjusted with 0.1% peptone water to an optical density (OD) at 630 nm of 0.7 (ca. 10^9 CFU/ml) and combined in equal volume to obtain a five-strain mixture prior to the inoculation of the manure. Cell populations of *E. coli* O157:H7 in the five-strain mixture were determined by plating on Tryptic soy agar (TSA; Becton Dickinson) plates containing 100 μ g of ampicillin/ml (TSA-A).

Soil (Tifton sandy loam soil) samples were collected from Tifton, Ga. A five-strain mixture of GFP-labeled *E. coli* O157:H7 cells were inoculated into cow manure by spraying 10 ml of the culture mixture with a spray bottle (Sprayco, Detroit,

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Mich.; sanitized by soaking in 70% ethanol) on the surface of 1000 g of manure and then mixing for 5 min with a 40-ml sterile spoon to homogeneously distribute the *E. coli* O157:H7. The inoculated manure was held at 23°C in sterile polypropylene trays (26 by 16 by 6.4 cm) supported by test tube racks in a plastic box (45 by 42 by 30 cm) with ca. 2.5 cm of tap water on the bottom to retain moisture in the manure. After 20 to 24 h of incubation, the inoculated manure was mixed with unautoclaved or autoclaved (121°C for 20 min, on three successive days) soil at a ratio of 1 part of manure to 10, 25, 50, or 100 (wt/wt) parts of soil, i.e., 1:10, 1:25, 1:50, or 1:100, respectively. The manure-amended soil was then held inside a polyethylene plastic box with lid (59 by 42 by 30 cm) at 21, 15, and 5°C.

Duplicate soil samples (ca. 25 g each) were obtained from each treatment tray at each sampling interval for determination of *E. coli* O157:H7 counts, pH, and moisture content. Each sample (5 g) was added to 45 ml of 0.1% peptone water in a sterile Whirl-Pak bag and macerated in a Seward Stomacher for 1 min at medium speed. Dilutions were prepared using 0.1% peptone water, and 0.1-ml portions of each dilution were spread onto TSA-A plates which were incubated at 37°C for 48 h for the determination of *E. coli* O157:H7 counts. The GFP-labeled *E. coli* O157:H7 colonies were counted under an UV light at 365 nm.

When *E. coli* O157:H7 could not be detected by the direct plating method (detection limit of 50 CFU/g), a selective enrichment culture method was used. A 1-g manure-amended soil sample was added to 99 ml of selective enrichment broth, i.e., TSB-A and incubated at 37°C for 24 h with agitation (150 rpm). Dilutions of cultures were surface plated on TSA-A plates. Randomly selected colonies that were green and fluoresced under UV light were confirmed as *E. coli* O157 by an *E. coli* O157 latex agglutination test (Oxoid Limited, Hampshire, England). The pH of manure-amended soil was determined by adding 5 g of soil to 250 ml of distilled water. The suspension was stirred for 5 min and then allowed to settle for 5 min. The pH of the liquid was determined with an Accumet Basic pH meter (Fisher Scientific). Moisture content of manure-amended soil was determined by drying 5 g of soil at 105°C for 24 h in a Precision oven (Precision Scientific) and then weighing the residual.

Microbiological data were converted to log CFU/gram for statistical analysis. An analysis of variance (ANOVA) for a completely randomized design with repeated measures across dates was conducted to determine if general differences existed among the four treatment means. Specific comparisons between any pair of treatment means at any date were accomplished with the Fisher's least-significant difference (LSD) test. All calculations were performed using the general linear model (GLM) procedure of the Statistical Analysis System (SAS 2001, Cary, N.C.). The analysis was repeated for each of the six temperature and soil conditions.

E. coli O157:H7 populations in manure-amended autoclaved soil at 5°C for 3 days decreased by 0.5 to 1.0 log₁₀ CFU/g compared to no change in manure-amended unautoclaved soil (Fig. 1A and B). During the following 3 weeks, *E. coli* O157:H7 populations in all samples decreased at approximately the same rate. However, during the initial 50 days, *E. coli* O157:H7 populations declined more rapidly in manure-amended autoclaved soil formulated at 1:50 and 1:100 than in samples formulated at 1:25 and 1:10. The pathogen survived for up to 77

days at 5°C in 1:10 samples. *E. coli* O157:H7 in manure-amended unautoclaved soil was inactivated during the initial 50 days more rapidly in combinations of 1:10 and 1:25 samples than in 1:50 and 1:100 samples. The pathogen was not detected by the 63rd day in manure-amended unautoclaved soil samples. At 15°C, *E. coli* O157:H7 populations within 7 days increased by ca. 0.5 to 2 log₁₀ CFU/g in manure-amended autoclaved soil, whereas populations declined by 3 log₁₀ CFU/g in manure-amended unautoclaved soil (Fig. 1C and D). During the following weeks, *E. coli* O157:H7 populations in manure-amended unautoclaved soil declined considerably more rapidly than in manure-amended autoclaved soil. In unautoclaved soil samples, *E. coli* O157:H7 populations decreased by 5 log₁₀ CFU/g within 42 days in samples of all four combinations and was no longer detected thereafter in the 1:10 samples. However, the pathogen was detected only by enrichment culture in samples formulated at 1:25, 1:50, and 1:100 for more than 109 days. *E. coli* O157:H7 survived in 1:25, 1:50, and 1:100 manure-amended autoclaved soil samples at 15°C for greater than 226 days, at which time there was a 3- to 4- log₁₀ CFU/g reduction in cell populations. *E. coli* O157:H7 in 1:10 samples of manure-amended autoclaved soil decreased to undetectable populations (>6 log₁₀ CFU/g reduction) at 152 days at 15°C. Similar survival patterns for *E. coli* O157:H7 were observed for the 1:25, 1:50, and 1:100 formulations of manure-amended autoclaved soil samples held at 21°C, with only a ca. 1.5 to 2 log₁₀ CFU/g reduction during 63 days of storage (Fig. 1E). The pathogen died off more rapidly in the 1:10 formulation, with a ca. 5 log₁₀ CFU/g reduction within 40 days (Fig. 1E). The pathogen died off more rapidly in manure-amended unautoclaved soil than in the autoclaved soil at 21°C (Fig. 1F). The pathogen was no longer detectable in 1:10, 1:25, 1:50, and 1:100 formulated samples at 117, 231, 180, and 145 days, respectively. Overall, *E. coli* O157:H7 survived longer in manure-amended unautoclaved soil held at 21°C than at 15°C. *E. coli* O157:H7 at 15 or 21°C, depending on the manure-to-soil ratio, survived for more than 226 days in manure-amended autoclaved soil (Table 1), whereas in manure-amended unautoclaved soil, the pathogen decreased to undetectable populations within 165 days at 15°C and within 231 days at 21°C (Fig. 1D and E).

The *E. coli* O157:H7 survival data in Fig. 1 were analyzed statistically across all dates to determine if general differences existed among the four treatment means. The LSD values ($P < 0.05$) between any pair of treatment means at any date were 0.9595, 0.4368, 0.3418, 0.786, 0.9498, and 0.9775 log CFU/g for Fig. 1A, B, C, D, E, and F, respectively. *E. coli* O157:H7 cells died off significantly ($P < 0.05$) more rapidly in 1:10 samples than in 1:25, 1:50, and 1:100 samples in autoclaved soil held at 15 and 21°C.

Tifton sandy loam soil, which had a pH of 7.0 and a 7% moisture content (MC) and contained P, K, Ca, and Mg at 140, 123, 724, and 85 lb/acre, respectively, was used for the study. After adding manure and *E. coli* O157:H7 to both autoclaved and unautoclaved soil, the 1:10 samples had the highest MC and pH, whereas the 1:100 samples had the lowest MC and pH at the onset. Over time, there was a gradual loss of moisture from all samples. The moisture content of the manure-amended soils, either autoclaved or unautoclaved, decreased to 0.6 to 3% by 68 days of storage. The initial aerobic plate

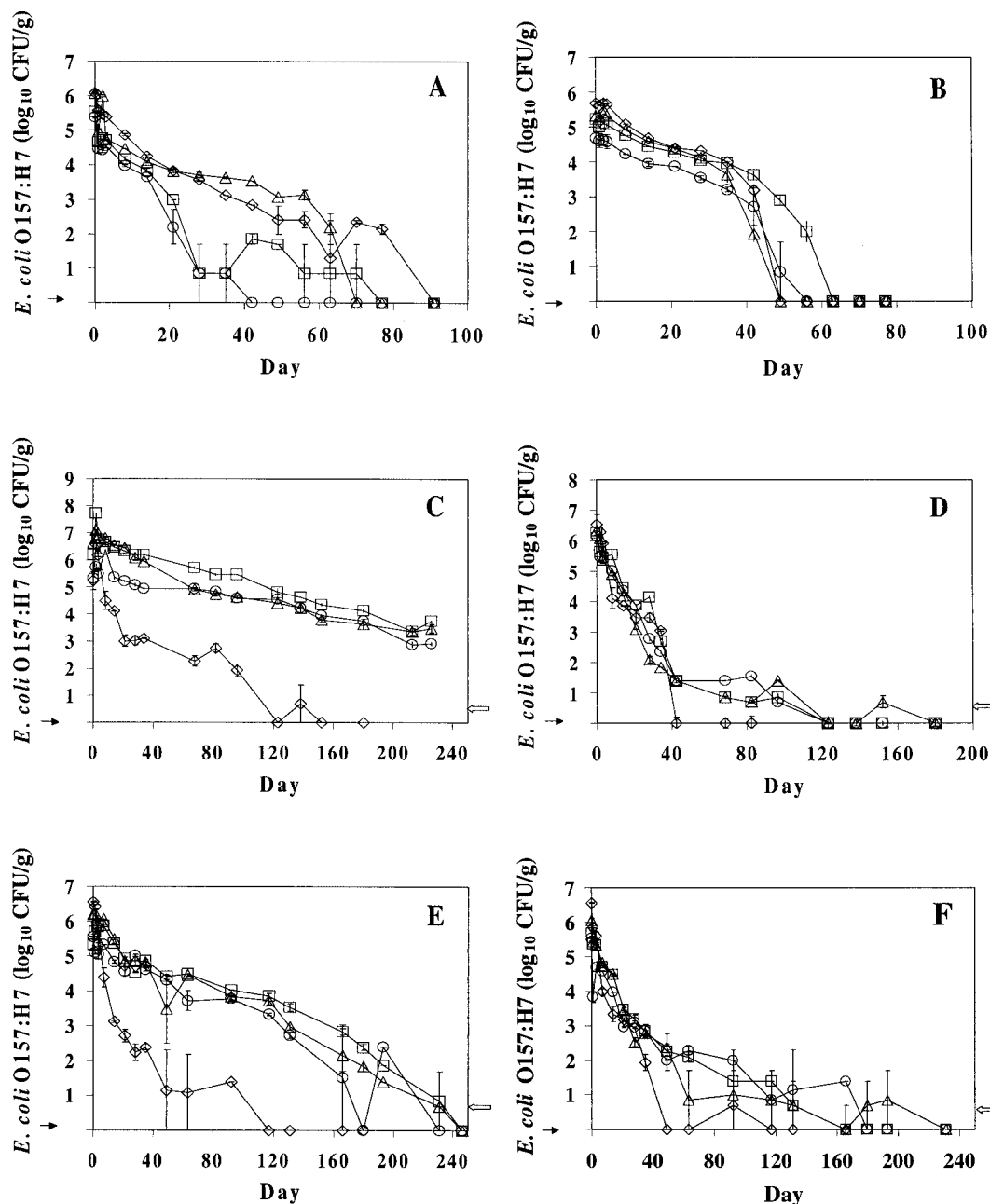


FIG. 1. Survival of *E. coli* O157:H7 at 5°C in manure-amended autoclaved (A) and unautoclaved (B) soil, at 15°C in manure-amended autoclaved (C) and unautoclaved (D) soil, and at 21°C in manure-amended autoclaved (E) and unautoclaved (F) soil. *E. coli* O157:H7 was enumerated on TSA-A plates from manure-to-soil formulations of 1:10 (◇), 1:25 (△), 1:50 (□), and 1:100 (○). →, not detected by enrichment culture; ←, detection by enrichment culture only. Each point represents the average of duplicate trials, and the bars at each point indicate the standard deviation of results obtained at each sampling time.

count in soil was 1.9×10^{10} CFU/g, and no ampicillin-resistant bacteria were detected. After storage at 21°C, the total aerobic plate counts in both autoclaved and unautoclaved manure-amended soil decreased steadily from ca. 10^{10} to ca. 10^7 CFU/g at 120 days and then remained at that level for up to 230 days.

During the first 2 weeks at 5°C, the pH of all manure-amended soil samples increased ca. 0.5 but thereafter decreased with increased storage time. The 1:10 manure-amended autoclaved soil retained a slightly higher pH than the

other soil samples. The pH of the manure-amended unautoclaved soil samples was lower than the autoclaved equivalents after storage for more than 2 months at 5°C. Apparently, the relatively higher pH in the soil favored the survival of *E. coli* O157:H7 at 5°C. The pH of manure-amended unautoclaved soil held at 15°C increased slightly during storage for 1 week and for 2 weeks in autoclaved soil. The pH change in manure-amended autoclaved or unautoclaved soil varied by approximately ± 1 pH unit during storage at 21°C. This variability

TABLE 1. Survival of *E. coli* O157:H7 in manure-amended autoclaved or unautoclaved soil held at different temperatures

Samples	Days of survival at storage temp (°C) of:		
	5	15	21
Manure:autoclaved soil			
1:10	77 ^a	138	103
1:25	63	>226	231
1:50	70	>226	231
1:100	35	>226	193
Manure:unautoclaved soil			
1:10	42	34	103
1:25	42	152	193
1:50	56	109	174
1:100	49	109	131

^a Maximum day at which *E. coli* O157:H7 was detected by either direct plating or enrichment culture methods.

could in part be attributed to variation in the distribution of manure-amended soil ingredients as well as variation that occurs at different locations during natural composting metabolic activities.

Properties of soil influencing the survival or inactivation of pathogenic bacteria include soil composition, pH, water activity, oxidation-reduction potential, presence of a rhizosphere, and microbial interactions (8, 16). Under field conditions, other variables, such as solar radiation and dryness, may also affect the survival of human pathogens. In our study, autoclaved soil was used as a control to minimize the influence of indigenous soil microorganisms on the survival and growth of *E. coli* O157:H7. Cow manure normally contains more than 10⁹ CFU of indigenous bacteria/g. In samples in which inoculated cow manure was mixed with autoclaved soil, ca. 10⁷ to 10⁸ CFU of manure bacteria/g were introduced into the autoclaved soil. The antagonistic effect of manure microorganisms was likely a factor in killing *E. coli* O157:H7 cells in our manure-amended soil studies at 15 and 21°C. *E. coli* O157:H7 cells in soil containing both plentiful manure nutrients and the highest population of manure microorganisms (1 part manure per 10 parts soil) died off more rapidly at growth temperatures in autoclaved soil than in soil formulations (1:25, 1:50, and 1:100) containing less manure microorganisms and nutrients. Other contributing factors may have included the pH and moisture content of the manure-amended soil, with the 1:10-formulated soil preparation maintaining conditions that were more favorable for growth of competitive manure microorganisms.

In addition to apparent antimicrobial activities of microorganisms in manure to *E. coli* O157:H7, indigenous soil microorganisms also appear to contribute to inactivation of *E. coli* O157:H7. *E. coli* O157:H7 was inactivated more rapidly in unautoclaved soil than in autoclaved soil at all three temperatures and with an equivalent amount of manure added. However, in unautoclaved soil, small numbers of the pathogen, which were only detected by enrichment culture, survived for very long periods of time at 15 and 21°C.

In the manure-amended autoclaved soil, *E. coli* O157:H7 populations increased from 0.3 to 2 log₁₀ CFU/g within 3 days at 15 and 21°C in manure-to-soil formulations of 1:25, 1:50, and 1:100. Gagliardi and Karns (10) also determined in a study

simulating conditions of rainfall that *E. coli* O157 cells grew well in soil cores that were treated with manure. However, *E. coli* O157:H7 growth did not occur in unautoclaved soil in our study, likely because of the antagonistic activity of soil bacteria that were present. Variability in the growth of *E. coli* O157:H7 that occurred in manure-amended soil of different types is likely attributed to variation in the antimicrobial activity of different microbes present in different soil.

Our study revealed that *E. coli* O157:H7 can survive for extended periods of time in manure-amended soil, even under very dry conditions, i.e., less than 1% moisture content in the soil. Maule (14) reported survival of *E. coli* O157:H7 for 130 days at 18°C when inoculated into a laboratory-prepared soil and grass microcosm. Fenlon et al. (8) applied *E. coli* O157:H7 cells (3 × 10¹ CFU/100 ml) inoculated in cattle slurry to arable and grass plots on a clay loam soil and determined that *E. coli* O157 was detected only in the soil and on the grass during the first week after land application. In contrast, Maule (13) observed prolonged survival of *E. coli* O157 with very high application rates of manure-containing *E. coli* O157 on soil cores with a grass cover.

We determined rates of inactivation of *E. coli* O157:H7 in soil containing different amounts of manure and held at different temperatures. Intensive application of manure to soil (e.g., 1 part manure to 10 parts soil versus 1:25, 1:50 or 1:100) generally resulted in greater inactivation of *E. coli* O157:H7 with a possible exception at 5°C, which is a temperature at which microorganisms antagonistic to *E. coli* O157:H7 may be less competitive because of slow or no growth or failure to produce significant amounts of antimicrobials against *E. coli* O157:H7. Microorganisms naturally occurring in soil appear to have a major influence on rates of inactivation of *E. coli* O157:H7 at 15 and 21°C, as the rates of pathogen inactivation were considerably less at these temperatures in manure-amended autoclaved soil than in manure-amended unautoclaved soil. Results from these studies provide useful information in identifying manure handling practices to reduce the risk of *E. coli* O157:H7 transmission to foods produced in the presence of animal waste. Additional studies that would be beneficial include identifying and characterizing the soil microorganisms that inactivate *E. coli* O157:H7 and further elucidating the environmental conditions that are optimal to kill pathogens in manure and soil.

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