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Reduction of *Escherichia coli* O157:H7 and *Salmonella enterica* Serovar Enteritidis in Chicken Manure by Larvae of the Black Soldier Fly

MARILYN C. ERICKSON,[1]* MAHBUB ISLAM,[1] CRAIG SHEPPARD,[2] JEAN LIAO,[1] AND MICHAEL P. DOYLE[1]

[1]*Center for Food Safety and Department of Food Science and Technology, University of Georgia, 1109 Experiment Street, Griffin, Georgia 30223; and* [2]*Department of Entomology, Coastal Plain Experiment Station, University of Georgia, Tifton, Georgia 31793, USA*

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ABSTRACT

Green fluorescent protein–labeled *Escherichia coli* O157:H7 and *Salmonella enterica* serovar Enteritidis were inoculated at 10^7 CFU/g into cow, hog, or chicken manure. Ten- or 11-day-old soldier fly larvae (*Hermetia illucens* L.) (7 to 10 g) were added to the manure and held at 23, 27, or 32°C for 3 to 6 days. Soldier fly larvae accelerated inactivation of *E. coli* O157:H7 in chicken manure but had no effect in cow manure and enhanced survival in hog manure. The initial pH values of the hog and chicken manure were 6.0 to 6.2 and 7.4 to 8.2, respectively, and it is surmised that these conditions affected the stability of the larval antimicrobial system. Reductions of *E. coli* O157:H7 populations in chicken manure by larvae were affected by storage temperature, with greater reductions in samples held for 3 days at 27 or 32°C than at 23°C. Pathogen inactivation in chicken manure by larvae was not affected by the indigenous microflora of chicken manure, because *Salmonella* Enteritidis populations in larvae-treated samples were approximately 2.5 log lower than control samples without larvae when either autoclaved or nonautoclaved chicken manure was used as the contaminated medium during 3 days of storage. Extending the storage time to 6 days, larvae again accelerated the reduction in *Salmonella* Enteritidis populations in chicken manure during the first 4 days of storage; however, larvae became contaminated with the pathogen. After 2 days of feeding on contaminated manure, *Salmonella* Enteritidis populations in larvae averaged 3.3 log CFU/g. Populations decreased to 1.9 log CFU/g after 6 days of exposure to contaminated chicken manure; however, the absence of feeding activity by the maggots in later stages of storage may be responsible for the continued presence of *Salmonella* Enteritidis in larvae. Transfer of contaminated larvae to fresh chicken manure restored feeding activity but led to cross-contamination of the fresh manure.

Animals serve as reservoirs of many significant foodborne pathogens, including *Escherichia coli* O157:H7 and *Salmonella* spp., and consequently their feces may also contain these organisms *(18, 22)*. Application of this animal waste to land as a crop fertilizer therefore increases the risk for horizontal transmission of the organism. Several studies have determined the stability of these organisms in different types of manure. Temperature, aeration, pH, and manure composition influence the rate of pathogen reduction during storage of manure *(7, 8, 10, 31)*.

To ensure that pathogens are eliminated from manure before application as a fertilizer, various treatments have been recommended. Composting is the most common treatment whereby manure is mixed with a carbon source (e.g., straw or sawdust) to facilitate biological oxidation of the materials *(12)*. This treatment system reduces the bulk of manure, making it more economical to transport the finished product off-farm. It also has the advantage that, when it is performed effectively, pathogenic bacteria are killed by the heat generated in the early phases of the composting. Unfortunately, it is difficult to create the right conditions to guarantee that sufficient heat (>50°C) for inactivation is applied throughout the compost pile for more than 1 week *(11)*. Likewise, temperatures generated by anaerobic sewage digesters are often too low to destroy all pathogens that may be present *(26)*. Chemical treatments that have proven successful in killing pathogens in manure include gassing with ammonia *(9)* and application of sodium carbonate and alkali *(1)*.

A novel approach under study is exploring the potential for generation of high-quality animal feedstuffs via digestion of manure by larvae of the nonpest black soldier fly (*Hermetia illucens* L.) *(23)*. In such systems, the mass of manure is reduced while converting residual protein and other nutrients in manure to self-collecting prepupae of 41 to 42% crude protein. Feeding studies with chickens *(6)*, pigs *(21)*, and catfish and tilapia *(3)* have shown that soldier fly larvae or larval meal was a suitable replacement for a high proportion of conventional protein and fat sources.

In the medical field, fly larvae (*Lucilia sericata* (Meigen)) have been reported to reduce or eliminate bacteria in infected wounds *(27)* through either ingestion and digestion of the bacteria or generation of antimicrobial compounds. Similarly, earthworms (*Eisenia foetida*) reduced *Salmonella enterica* serovar Enteritidis populations in horse manure *(20)*. In laboratory studies, Simmons *(25)* collected medicinal larval secretions and demonstrated their ability to kill

* Author for correspondence. Tel: 770-412-4742; Fax: 770-229-3216; E-mail: mericks@griffin.uga.edu.

Staphylococcus aureus, hemolytic and nonhemolytic streptococci, *Clostridium welchii, Proteus vulgaris,* and *Eberthella typhi*. More recently, Mumcuoglu et al. *(19)* visualized green fluorescent protein (GFP)–producing *E. coli* in the alimentary canal of fly larvae and found decreasing intensity with passage through the digestive tract. A battery of defense proteins synthesized by insects in response to bacterial challenge may be responsible. Cecropins, lytic proteins, have been isolated from larvae of blowflies (*Calliphora vicina*) *(4)*, whereas defensins, 3- to 4-kDa peptides that exert broad-spectrum antimicrobial activities through membrane permeation, have been isolated from the midgut of the blood-sucking fly *Stomoxys calcitrans (16)*. Another antibacterial substance, *p*-hydroxycinnamaldehyde, isolated from the larvae of the saw fly, *Acantholyda parki* S., was also found to have a broad antibacterial spectrum against both gram-negative and gram-positive bacteria *(15)*. Based on these findings, similar antibacterial substances might be present in soldier fly larvae. This investigation therefore sought to determine the ability of soldier fly larvae to reduce the foodborne pathogens *E. coli* O157:H7 and *Salmonella enterica* serovar Enteritidis in manure.

MATERIALS AND METHODS

Preparation of pathogens for inoculation into manure. Five strains of *E. coli* O157:H7 (strains E0143, C7927, K262, C0083, and E0139) and one strain of *Salmonella enterica* serotype Enteritidis (ME 18) were labeled with jellyfish GFP according to the method described by Jiang et al. *(11)*. Transformed strains were subsequently selected from isolated colonies grown on Luria-Bertani (Difco Laboratories, Sparks, Md.) plates containing 100 μg/ml of ampicillin (Sigma Chemical Co., St. Louis, Mo.). When viewed under a handheld UV light (365 nm), the resulting ampicillin-resistant transformed colonies emitted bright green fluorescence.

Each strain of *E. coli* O157:H7 or *Salmonella* was individually inoculated into 10 ml of tryptic soy broth (Becton Dickinson, Sparks, Md.) containing 100 μg/ml of ampicillin (TSB-A). Following incubation (37°C, 16 to 18 h) with agitation (150 rpm), 0.5 ml of each isolate suspension was transferred to 100 ml of TSB-A and incubated again (37°C, 16 to 18 h) with agitation (150 rpm). The bacteria were harvested by centrifugation (4,000 × *g*, 20 min, 4°C) and washed three times in 0.1% peptone water (Difco). The individual strains were reconstituted with 0.1% peptone water to an optical density of 0.5 (approximately 10^8 CFU/ml). Equal volumes of *E. coli* O157:H7 strains were combined to give one five-strain mixture. Cell populations of the *E. coli* O157:H7 mixture and *Salmonella* strain were determined by plating on tryptic soy agar (Difco) containing 100 μg/ml of ampicillin (TSA-A).

Collection and treatment of manure. Fresh cow manure was collected from a dairy farm near Griffin, Ga., and frozen for at least 24 h to kill insect eggs. A similar treatment was applied to fresh hog manure and hen manure procured from The University of Georgia's Experiment Station in Tifton, Ga. Before inoculation of the manure with pathogens, thawed manure was assayed for aerobic plate count (37°C, 24 h) by serial dilution (1:10) of manure on TSA. To reduce indigenous microflora populations, portions of manure (1,500 g) were autoclaved for 45 min at 121°C. Inoculation of autoclaved and nonautoclaved manure was accomplished by spraying (Sprayco, Detroit, Mich.; head sanitized by spraying 50 ml of 70% ethanol through head) 9 ml of the pathogen culture (*E. coli* O157:H7 mixed culture or *Salmonella* culture) onto the surface of the manure (900 g). Control samples of manure had an equal volume of deionized water sprayed on them. The pathogen was distributed throughout the manure sample by mixing the manure for 5 min with a 40-ml sterile spoon.

Experimental setup. Soldier fly larvae (*Hermetia illucens* L.) were reared from eggs for 10 to 11 days according to conditions established by Sheppard et al. *(24)*. Larvae were removed from the rearing media and weighed before adding 7 or 10 g to plastic, 16-oz (473-ml) food cups (Sweetheart Cup Co., Chicago, Ill.) containing 50, 75, or 125 g of manure. Another set of cups containing equivalent amounts of manure without larvae served as the control. Each sample cup was then covered with a paper towel before storing at 23, 27, or 32°C. For each replicate trial, manure was removed after 0, 1, 2, and 3 days of storage from three control and three treated sample cups and manure was assayed for pathogen populations, pH, and moisture content. On the last day of storage, larvae were separated from treated manure samples and weighed. In selected trials, pathogen populations were also determined in larvae.

A separate set of experiments was conducted during a 6-day period of storage at 27°C. In this case, both manure and larvae were sampled from control and treated cups in triplicate at each of the sampling days (0, 2, 4, or 6). Larval weight was recorded and pathogen populations in both manure and larvae were determined separately at each sampling period. Manure was also assayed for moisture content and pH.

Enumeration of pathogens. Manure samples (1 g) were mixed with 9 ml of 0.1% peptone water in a sterile Whirl-Pak bag and macerated in a stomacher for 1 min at medium speed. Dilutions were prepared using 0.1% peptone water, and 0.1-ml portions of each dilution were surface plated onto TSA-A plates. Pathogen counts were determined on these plates under UV light (365 nm) following incubation of plates at 37°C for 48 h.

When pathogens could not be detected by the direct plating method, a selective enrichment method was used. In this method, manure (1 g) was added to 9 ml of TSB-A and mixed. From this mixture, 0.5 ml was removed and added to 100 ml of TSB-A for incubation at 37°C for 24 h with agitation (150 rpm). Dilutions of this incubated culture were surface plated on TSA-A. Colonies that fluoresced green under UV light were confirmed either as *E. coli* O157:H7 colonies by an *E. coli* O157 latex agglutination test (Oxoid Ltd., Hampshire, UK) or as *Salmonella* colonies by a *Salmonella* latex agglutination kit (Oxoid).

Analyses of pH and moisture in manure. The pH of manure samples was determined by adding 5 g to 50 ml of deionized water. The pH was measured using an Accumet Basic pH meter (Fisher Scientific, Pittsburgh, Pa.). Moisture content of manure samples was determined by drying manure (1 g) at 105°C for 24 h in a Precision oven (Precision Scientific, Winchester, Va.) and determining residual weights.

Statistical analyses. Bacterial populations were converted to log CFU/g before statistical analysis. Data were analyzed by analysis of variance using the StatGraphics Plus software package (Manugistics, Rockville, Md.), and where statistical differences were noted ($P < 0.05$), differences among sample means were determined using the least significant difference test. Number of replicate independent trials (n) are specified with each experimental set of data.

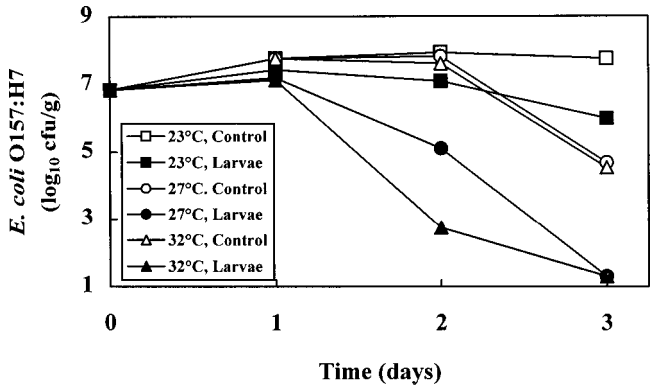

FIGURE 1. *Influence of temperature on inactivation of* E. coli *O157:H7 in chicken manure by soldier fly larvae. Each point represents the mean of two independent trials (*n = *2). Each sample consisted of 125 g of manure. In treated samples, 7 g of 11-day-old soldier fly larvae was added.*

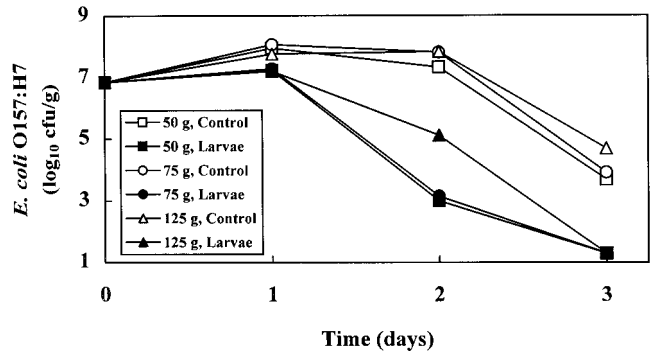

FIGURE 2. *Soldier fly larvae inactivation of* E. coli *O157:H7 in different ratios of chicken manure:larvae. Each point represents the mean of two independent trials (*n = *2). Samples contained 50, 75, or 125 g of chicken manure and were held at 27°C. Treated samples contained 7 g of 11-day-old soldier fly larvae.*

RESULTS AND DISCUSSION

Optimization of environmental conditions for inactivation of *E. coli* O157:H7 in chicken manure by soldier fly larvae. Chicken hen manure contaminated with *E. coli* O157:H7 (approximately 10^7 CFU/g) was held for a period of 3 days at temperatures ranging from 23 to 32°C. In the absence of larvae (control), *E. coli* O157:H7 populations increased slightly during storage when held at 23°C (Fig. 1). Storage at higher temperatures (27 or 32°C), however, led to a 2-log reduction in control pathogen populations between 2 and 3 days. Such rates of inactivation are comparable to those previously reported for this pathogen in chicken manure *(10)*. In the presence of larvae, however, inactivation of pathogens was accelerated compared with control, with a 1.5-log and a 5-log reduction in *E. coli* O157:H7 populations by day 2 in 27 and 32°C samples, respectively. Following 3 days of storage, *E. coli* O157:H7 could not be detected on enrichment in either the 27 or 32°C larval samples.

Larval growth was also monitored during these temperature studies to ensure that biomass conversion of manure to a higher valued feedstuff would not be sacrificed. The greatest growth of soldier fly larvae in chicken manure occurred in samples held at 27°C, with a 67% increase in larval weight by day 3 (Table 1). Growth of larvae in hog and cow manure was also optimal at 27°C; however, in the case of hog manure, the weight gain at 27°C was not statistically different from weight gains at 32°C.

Inactivation of *E. coli* O157:H7 by soldier fly larvae was similar in systems containing either 50 or 75 g of chicken manure (Fig. 2). In systems containing 125 g of chicken manure, inactivation of pathogens was slightly slower than occurred in 50- or 75-g systems in that a 2-log difference in population levels for these systems was observed on day 2. Although the percentage moisture decreased slightly in control systems containing either 50 or 75 g of chicken manure, the changes were not statistically significant (Fig. 3).

Fate of *E. coli* O157:H7 populations in different manures with soldier fly larvae. The fate of *E. coli* O157:H7 in the presence of soldier fly larvae depended on the type of manure (Fig. 4). Although larvae markedly accelerated inactivation of pathogens in chicken manure, no statistical differences in pathogen populations were observed in 0- and 3-day stored dairy cow manure in either the presence or absence of larvae. An increase in pathogen cell

TABLE 1. *Growth of soldier fly larvae in manure held 3 days at different temperatures*[a]

Temperature (°C)	Maggot weight gain (g)		
	Chicken manure[b]	Hog manure[b]	Cow manure[c]
28	3.09 ± 1.58	4.14 ± 0.62	2.20
27	4.72 ± 0.23	11.06 ± 2.47	4.27
32	4.24 ± 0.26	10.92 ± 2.62	4.00

[a] Initial weight of larvae was 7 g. Each sample contained 125 g of manure.

[b] Each value represents the mean ± SEM of two independent trials (n = 2).

[c] Each value represents the average weight gain obtained in one independent trial (n = 1).

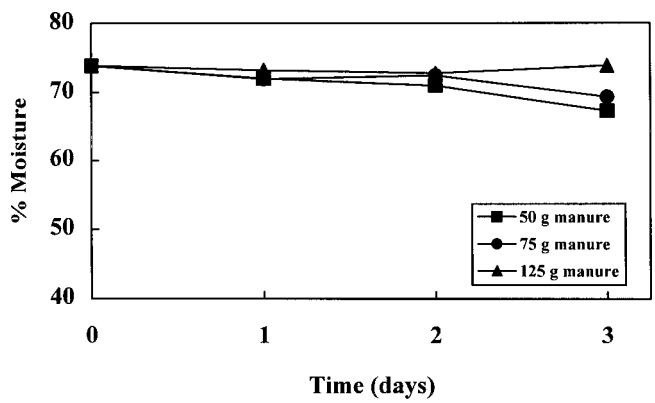

FIGURE 3. *Moisture loss during storage of variable quantities of chicken manure. Each point represents the mean of two independent trials (*n = *2). No samples contained larvae. All samples were stored at 27°C.*

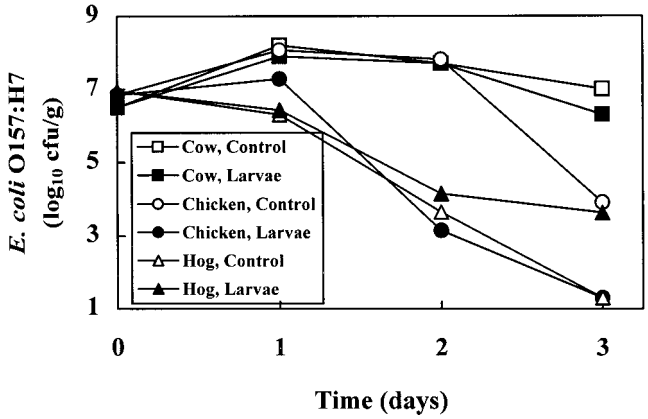

FIGURE 4. *Reduction of* E. coli *O157:H7 by soldier fly larvae in different types of manure. Two independent trials were conducted for both chicken and hog manure* (n = *2), whereas only one trial* (n = *1) was conducted for cow manure systems. All samples contained 75 g of manure, and treated samples also contained 7 g of soldier fly larvae. Samples were stored at 27°C.*

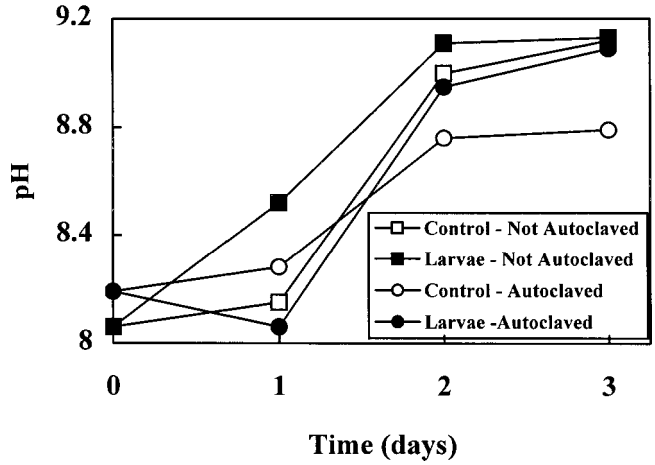

FIGURE 5. *Influence of indigenous microflora on pH of stored chicken manure. Each point represents the mean of six different trials* (n = *6). All samples contained 75 g of chicken manure and were held at 27°C. Treated samples contained either 7 or 10 g of 10- or 11-day-old soldier fly larvae.*

numbers during the first 2 days is consistent with the population increases observed by Wang et al. *(31)* during the first 2 days of storage of untreated cow manure. In that study, a 3-log and 5-log (CFU/g) inactivation of the pathogen required 42 and 49 days (22°C) or 49 and 56 days (37°C), respectively. Similarly, Himathongkham et al. *(8)* determined *E. coli* O157:H7 survived well in untreated dairy manure, with decimal reduction times (90% reduction) at 37°C ranging from 3.58 to 8.91 days, depending on the location of the pathogen within the manure. At 23°C, 14 days of storage were needed to observe decreases in *E. coli* O157:H7 below the preincubation level of 3.6×10^7 CFU/g *(14)*.

Larval growth in hog manure was more than double the growth achieved in chicken manure (Table 1), yet *E. coli* O157:H7 populations were higher in hog manure when larvae were present than when they were absent (Fig. 4). The different larval responses in hog and chicken manure samples may reflect the differences in the composition of the manure. The initial pH of hog manure ranged from 6.0 to 6.2, whereas the initial pH of chicken manure ranged from 7.4 to 8.2. In addition, the pH of hog manure decreased during storage, whereas the pH of chicken manure increased. Hence, stabilities of activators or inhibitors of soldier fly larvae would likely vary in hog and chicken manure if they were pH dependent. For example, maximal stability of defensin A, an inducible antibacterial protein isolated from the larvae of the fleshfly *Phormia terranovae,* is near its isoelectric point (8.3) *(17)*.

Contribution of indigenous microflora in chicken manure to inactivation of *Salmonella enterica* serovar Enteritidis by soldier fly larvae. Exposure of chicken manure to heat treatment under pressure (121°C, 45 min) reduced the aerobic bacterial count in chicken manure from 8.7 log CFU/g to 3.1 log CFU/g. Subsequently, different pH profiles of chicken manure were observed during storage for the autoclaved and nonautoclaved chicken manure (Fig. 5). In the absence of larvae, the pH of nonautoclaved chicken manure increased significantly more rapidly than that of autoclaved chicken manure. Increases in pH have been attributed to the production of ammonia by indigenous bacteria in the manure. Reduced amounts of ammonia produced may be the result of having a smaller population of aerobic bacteria in autoclaved manure to generate ammonia. In the presence of larvae, however, significant pH differences between autoclaved and nonautoclaved chicken manure were only observed on day 1. Thereafter, the pH levels of autoclaved and nonautoclaved chicken manure samples were similar. Nitrogenous excretory products, including ammonia, have been detected in samples containing antlion larvae *(30)*. Likewise, the generation of ammonia by soldier fly larvae could have contributed to a greater change in pH in samples that contained the larvae than was observed in autoclaved samples without larvae. Ammonia has a bactericidal effect on *Salmonella (9, 29)*. Hence, it was not surprising that *Salmonella* populations in the more alkaline control (nonautoclaved) samples were significantly lower than populations in the autoclaved chicken manure samples (Fig. 6). To factor out the variable response of control autoclaved and nonautoclaved samples, inactivation attributed to the presence of soldier fly larvae was expressed as the difference in *Salmonella* populations between control and larvae samples (Fig. 7). With this adjustment, on any given day, no statistical differences were observed in *Salmonella* cell numbers between autoclaved and nonautoclaved samples, indicating that the indigenous bacterial flora of chicken manure had little influence on the elimination of pathogens by larvae.

Contamination of soldier fly larvae with *Salmonella enterica* serovar Enteritidis. *Salmonella* populations in larvae following a 3-day exposure to contaminated chicken manure at 27°C ranged from nondetectable by enrichment to 7.0 log CFU/g larvae. The level of contamination in the larvae was dependent on whether chicken manure was autoclaved or not. In larvae exposed to nonautoclaved chicken

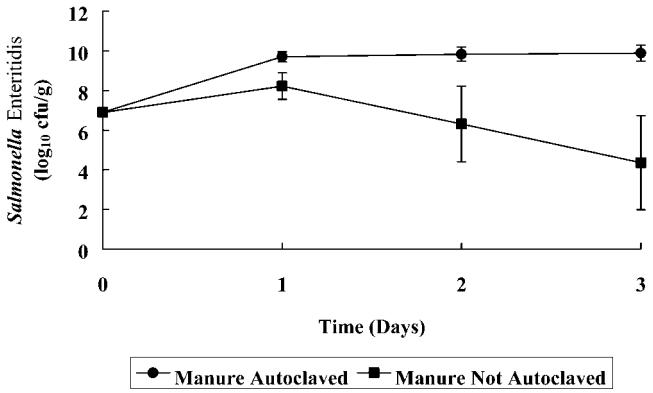

FIGURE 6. *Influence of indigenous microflora on* Salmonella *Enteritidis populations in stored chicken manure. Each point represents the mean of six independent trials (*n = 6*). Error bars denote the standard error of the means. Samples contained 75 g of either autoclaved or nonautoclaved chicken manure and were stored at 27°C.*

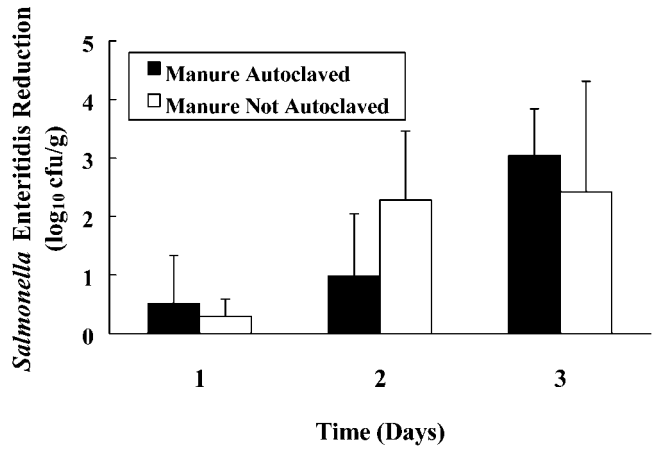

FIGURE 7. *Reduction in* Salmonella *Enteritidis populations attributed to soldier fly larvae in autoclaved and nonautoclaved chicken manure. Each bar represents the mean of six independent trials (*n = 6*), each trial being calculated from the net difference in populations of control samples and larvae-treated samples. Error bars denote the standard error of the means. All manure samples (75 g) were held at 27°C. Treated manure contained either 7 or 10 g of 10- or 11-day-old larvae.*

manure, *Salmonella* populations were 2.72 ± 1.38 log CFU/g, whereas larvae exposed to autoclaved chicken manure were 6.07 ± 0.89 log CFU/g. Transfer of contaminated larvae to fresh chicken manure facilitated elimination of pathogens from larvae but led to cross-contamination of the fresh manure (data not shown).

To provide further insight into *Salmonella* contamination of larvae, another study was conducted whereby larvae were exposed to contaminated chicken manure for 6 days. After 2 days, *Salmonella* populations in larvae-free chicken manure had increased slightly, whereas populations in chicken manure containing larvae had decreased by 4 log CFU/g from initial levels (Table 2). At the same time, *Salmonella* populations in the larvae were nearly 1 log higher than they were in the chicken manure. After 4 days of exposure of larvae to contaminated chicken manure, *Salmonella* levels in both chicken manure and larvae decreased from the 2-day exposed levels. Between 4 and 6 days of storage, however, no further decrease in *Salmonella* populations was observed in chicken manure or larvae. During this same period, larval mass decreased, suggesting that larval feeding was minimal or nonexistent. Such a change in activity is in agreement with previous observations that larval growth is diminished in older manures compared with fresh manures *(2)* and has been attributed to waste accumulation and unfavorable moisture *(13, 28)*. The absence of growth concurrently with loss of inactivation, however, suggests that inactivation is linked to actively growing larvae.

Assessment of the effectiveness in using soldier fly larvae to reduce pathogen populations in manure. To avoid cross-contamination of crops when manure is used as a fertilizer, elimination of pathogens from the manure must be ensured. In this study, application of soldier fly larvae to chicken manure has been found to accelerate the inactivation of *Salmonella* and *E. coli* O157:H7 in alkaline chicken manure. The greater pathogen loads found in slightly acidic stored hog manure containing larvae, however, suggest that the antimicrobial activity of larvae is only operable under alkaline conditions. These conclusions contrast to the findings of Greenberg *(5)*, who reported a 5-log destruction of *Salmonella, Proteus,* and *Streptococcus* spp. in less than 10 min in the maggot acid midgut. In this study, most of the inactivation attributed to larvae occurs within 1 to 3 days after larval exposure to the chicken manure. During this period, larvae are actively growing by ingesting

TABLE 2. Salmonella *Enteritidis populations in chicken manure and larvae held at 27°C for 6 days*[a]

	Salmonella Enteritidis populations (log CFU/g)			
	Manure[b]			
Day	Without larvae	With larvae	Larvae[c]	Larvae wt (g)
2	7.31 ± 0.77 C	2.41 ± 1.05 B	3.33 ± 1.04 B	13.9 ± 0.6 AB
4	2.83 ± 1.02 B	1.42 ± 0.28 C	1.89 ± 0.54 A	14.9 ± 1.0 B
6	1.65 ± 0.38 A	1.53 ± 0.36 A	2.15 ± 0.85 A	13.4 ± 1.2 A

[a] Each value represents the means ± SEMs of two independent trials ($n = 2$). Values in a column followed by the same letter are not significantly different ($P > 0.05$).

[b] Initial *Salmonella* Enteritidis populations in chicken manure were 6.92 ± 0.15 log CFU/g.

[c] Initial weight of soldier fly larvae was 10 g.

the manure and pathogens. Pathogen inactivation in the alimentary tract of larvae is therefore a likely scenario. Support for this idea is found in the study of Mumcuoglu et al. *(19)*, who demonstrated the destruction of bacteria in the alimentary tract of *Lucilia sericata* larvae. Inactivation does not appear to be immediate in the black soldier fly, because the concentration of pathogens in larvae becomes greater than it is in chicken manure. Transfer of contaminated larvae to fresh chicken manure restores feeding activity and inactivation of pathogens, but cross-contamination of the fresh manure also may occur. Pathogen reductions effected by soldier fly larvae in this study were significant but less than required to ensure absolute safety of chicken manure as a crop fertilizer. Systems supporting more vigorous larvae growth may provide greater pathogen reductions.

ACKNOWLEDGMENTS

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