Black Soldier Fly (Diptera: Stratiomyidae) Larvae Reduce *Escherichia coli* in Dairy Manure

QIAOLIN LIU,[1,2] JEFFERY K. TOMBERLIN,[2,3] JEFF A. BRADY,[4] MICHELLE R. SANFORD,[2] AND ZINIU YU[1]

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ABSTRACT *Escherichia coli* labeled with a green fluorescent protein was inoculated into sterile dairy manure at 7.0 log cfu/g. Approximately 125 black soldier fly larvae were placed in manure inoculated and homogenized with *E. coli*. Manure inoculated with *E. coli* but without black soldier fly larvae served as the control. For the first experiment, larvae were introduced into 50, 75, 100, or 125 g sterilized dairy manure inoculated and homogenized with *E. coli* and stored 72 h at 27°C. Black soldier fly larvae significantly reduced *E. coli* counts in all treatments. However, varying the amount of manure provided the black soldier fly larvae significantly affected their weight gain and their ability to reduce *E. coli* populations present. For the second experiment, larvae were introduced into 50 g manure inoculated with *E. coli* and stored for 72 h at 23, 27, 31, or 35°C. Minimal bacterial growth was recorded in the control held at 35°C and was excluded from the analysis. Black soldier fly larvae significantly reduced *E. coli* counts in manure held at remaining temperatures. Accordingly, temperature significantly influenced the ability of black soldier fly larvae to develop and reduce *E. coli* counts with greatest suppression occurring at 27°C.

KEY WORDS black soldier fly larvae, *Hermetia illucens*, Stratiomyidae, *Escherichia coli*, dairy manure

Demand for products from confined animal facilities can be expected to increase as the human population continues to grow. Waste production can also be expected to increase as livestock production continues to expand. Presently, 700,000 metric tons of broiler waste is produced annually on a worldwide basis (Turnell et al. 2007). Approximately 1.22 billion tons of cattle manure is produced annually in the United States (Islam et al. 2005), with production being concentrated in areas with high populations of confined animal facilities. For example, manure production by ≈40,000 dairy cattle in the Bosque River Watershed in Erath County, TX results in approximately one million metric tons annually (Bekele et al. 2006). Consequently, potential pollution of water sources, soil, and air by wastes produced in confined animal facilities is a concern for many of these areas.

Various methods have been proposed to manage dairy wastes. Land applications of manure have served as the primary method for waste management in the past; however, applications to fields have been identified as the primary nonpoint source for soluble phosphorus (Bekele et al. 2006). Consequently, more sustainable methods for handling manure from confined animal facilities have been studied. Composting is a common method used to recycle and manage solid wastes (Turner et al. 2005), such as animal manure (Cekmecelioglu et al. 2005). Manure is mixed with a carbon source to enhance oxidation (Erickson et al. 2004). This process results in a reduction of bulk material with remaining compost being used as fertilizer for crops such as wheat (Butler and Muir 2006).

Animal manure is known to contain pathogens such as *Escherichia coli* O157:H7 (Fremaux et al. 2006, Williams et al. 2006). Composting results in high temperatures (50–70°C) that will suppress *E. coli* O157:H7; however, the inability to maintain these high temperatures could result in their survival (Williams et al. 2006). Therefore, the potential for microbial contamination of soils and associated vegetation caused by the application of inadequately composted manure is a concern (Cekmecelioglu et al. 2005).

Black soldier fly, *Hermetia illucens* L., (Diptera: Stratiomyidae) larvae have been proposed as a method to reduce livestock manure (Sheppard et al. 1994). They significantly reduce accumulated dry matter in poultry (Sheppard et al. 1994) and dairy manure (Myers et al. 2008). Black soldier fly larvae reduce available phosphorus and nitrogen by 61–70 and 30–50%, respectively, in dairy manure (Myers et al. 2008). Additional benefits include suppression of house fly, *Musca domestica* L. (Diptera: Muscidae), populations by 94–100% in poultry manure (Sheppard

[1] State Key Laboratory of Agricultural Microbiology, National Engineering Research Center of Microbe Pesticide, Huazhong Agricultural University, Wuhan, Hubei, China.
[2] Corresponding author, e-mail: jktomberlin@ag.tamu.edu.
[3] Department of Entomology, 2475 Texas A&M University, College Station, TX.
[4] Texas A&M AgriLife Research, Stephenville, TX.

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1983). Bradley and Sheppard (1984) determined in the laboratory that oviposition in poultry manure by individuals from a wild house fly strain was reduced by 97% when black soldier fly larvae were present. Furthermore, black soldier fly prepupae can be self-harvested and used as a feed additive for swine (Newton et al. 1977), poultry (Hale 1973), and fish (St. Hilaire et al. 2007).

Black soldier fly larvae reduce pathogen loads in manure. Erickson et al. (2004) determined black soldier fly larvae can suppress *E. coli* O157:H7 and *Salmonella enterica* serotype Enteritidis (ME 18) populations in poultry manure (Erickson et al. 2004). They also examined the ability of black soldier fly larvae to reduce these pathogens in dairy cow and hog manure. However, their data indicated that the larvae had no effect on the pathogen populations in cow manure and augmented survivorship in hog manure. The purpose of this study was to determine the ability of black soldier flies to reduce *E. coli* populations in dairy manure.

Materials and Methods

Source of Black Soldier Fly Larvae and Bacteria. Neonate black soldier fly larvae were obtained from a colony maintained at the Texas A&M AgriLife Research Center, Stephenville, TX. Neonate larvae were reared for 15 d at 27°C on a (20% corn meal, 30% alfalfa meal, 50% wheat bran) Gainesville diet (Hogsette 1985). Approximately 125 larvae (10 g) were used in each replicate of each experiment. Larvae were placed in sterile manure immediately after its inoculation and homogenization with *E. coli.*

Collection and Treatment of Manure. Approximately 100 kg of dairy manure <6 h old were collected from a 1,000-head *Bos taurus* L. dairy operation in Stephenville, TX. This local dairy is a commercial production facility where cows are fed a standard, total mixed ration diet ad libitum. Between 1- and 5-kg manure samples were placed individually in 3.8-liter Ziploc bags and stored in a −20°C Kenmore Elite heavy duty commercial chest freezer (Sears, Roebuck and Company, Chicago, IL) for 1 wk before use. Manure was thawed to room temperature before being used in the experiments. Five hundred-milliliter glass jars were used as containers for these experiments. Each jar was inoculated with manure from a single bag. Consequently, a bag of manure constituted a replicate. Glass jars containing manure were autoclaved at 121°C for 45 min to reduce indigenous microflora populations (Erickson et al. 2004). Manure sterility before inoculation with bacteria was confirmed by dilution plating. Manure used in the experiment had a moisture content of ≈72%.

***Escherichia coli* Source.** To facilitate bacterial culturing and counting, *E. coli* ER2566 (New England Biolabs, Ipswich, MA) was transformed with the plasmid pQBI63 (Qbiogene, Irvine, CA). ER2566 is a nonpathogenic laboratory bacterial strain containing a T7 RNA polymerase gene, whereas the pQBI63 plasmid contains an ampicillin resistance gene, a pUC origin of replication, and a green fluorescent protein gene controlled by a lac repressor/T7 promoter.

***Escherichia coli* Growth Conditions and Counting.** Before inoculating manure samples, the bacteria were grown at 37°C in Luria-Bertani (LB) broth containing 100 μg/ml ampicillin and 0.5 mM Isoprpyl Beta Thiogalactopyranoside (IPTG) to an OD_{600} normalized to 0.3 with sterile 0.85% physiological saline. Bacterial cfu/ml before inoculation was determined by dilution plating. Bacteria were added to sterile manure at 1:100 (volume:weight) and mixed with a sterile spoon immediately before addition of fly larvae. After the treatment period, 10 g of dairy manure was put in a 250-ml Erlenmeyer flask containing 90 ml of sterile 0.85% physiological saline and placed on a shaker for 15 min with agitation (200 rpm). The number of *E. coli* remaining was determined by dilution plating of the manure sample. Bacteria were grown at 37°C for 17–18 h on LB agar plates containing 100 μg/ml ampicillin and 0.5 mM IPTG before counting. Single colonies emitted bright green fluorescence when observed with visible blue light on a Dark Reader DR195M Transilluminator (Clare Chemical Research, Dolores, CO).

Experimental Design. Two experiments examining the ability of black soldier fly larval density in combination with temperature to reduce *E. coli* counts in dairy manure were conducted. *E. coli* data were converted into log cfu/g before statistical analysis.

A preliminary study was conducted to determine the time interval needed to determine whether black soldier fly larvae reduce *E. coli* populations in dairy manure. Fifty grams of dairy manure was introduced into each of three mason jars, sterilized, inoculated with *E. coli*, and placed in Percival growth chambers at 27°C, 60–70% RH, and a photoperiod of 16:8 (L:D) h for 48 and 72 h. *E. coli* counts were made after these two incubation intervals. Levene's test for equality of variance indicated that the *E. coli* counts violated the assumption ($F = 10.484$; df = 7,16; $P = 0.007$) of analysis of variance (ANOVA). Therefore, the data were analyzed using the Kruskal-Wallis test (SPSS 2005). *E. coli* counts were not significantly different ($\chi^2 = 3.857$, df = 1, $P > 0.05$) between manure with and without black soldier fly larvae 48 h after initiating the experiment; however, a significant ($\chi^2 = 4.355$, df = 1, $P < 0.05$) reduction in the *E. coli* population was measured in manure with black soldier fly larvae rather than manure without larvae 72 h after initiating the experiment. Therefore, both experiments described below were conducted for 72 h.

Black Soldier Fly Larval Density. Either 50, 75, 100, or 125 g of dairy manure was added to each glass mason jar and sterilized. All jars were inoculated with *E. coli* and homogenized manually. Three replicates of each manure amount were inoculated with 10 g of black soldier fly larvae. Three replicates were not inoculated with larvae and served as controls. Replicates were covered with foil and stored at 27°C, 60–70% RH, and a photoperiod of 16:8 (L:D) h in an E-30B Percival growth chamber (Percival, Boone, IA). *E. coli* counts were determined 72 h after placement in the incuba-

tor. Larvae were removed from each replicate in each experiment after 72 h and weighed to determine weight gain. Data were analyzed using an ANOVA (SPSS 2005). A least significant difference (LSD) test (SPSS 2005) was used following a significant F test ($P < 0.05$) to separate mean weight gain differences.

To satisfy the assumption of equality of variance, the data were transformed using the $ln(x + 1)$ transformation before analysis with the univariate ANOVA procedure (SPSS 2005). Factors in the analysis included the presence of soldier flies, the amount of manure, and the interaction between these two factors on the number of *E. coli* colonies present at the conclusion of the study. After statistically significant results at the $\alpha = 0.05$ level, post hoc analyses were conducted with Fisher LSD among means to determine which manure amounts had significantly different in *E. coli* counts.

Effects of Temperature. Fifty grams of dairy manure was introduced into mason jars, sterilized, and inoculated with *E. coli* and placed in Percival growth chambers at 23, 27, 31, and 35°C, 60–70% RH, and a photoperiod of 16:8 (L:D) h for 72 h. Three replicates of each manure amount were inoculated with 10 g of black soldier fly larvae. Three replicates were not inoculated with larvae and served as controls.

Results indicated zero larval or bacterial growth in cultures raised at the highest temperature (35°C); thus, this portion of the data set was removed to aid in satisfying the assumptions of equality of variance. The dataset was also subjected to the $ln(x + 1)$ transformation to satisfy this assumption of ANOVA. The final transformed data set was analyzed with the univariate ANOVA procedure (SPSS 2005) with the factors of temperature, the presence of soldier flies, and the interaction between the two on the number of *E. coli* colonies at the conclusion of the study. Fisher LSD test was used for post hoc analysis of the mean number of *E. coli* colonies among the different temperatures. Statistical significance in *E. coli* counts between the treatments and controls was also observed at the $\alpha = 0.05$ level.

Results and Discussion

Previous research indicated black soldier fly larvae feeding on poultry manure deactivated *E. coli* OH157:H7 (Erickson et al. 2004). Their research also indicated that black soldier fly larvae could not reduce *E. coli* OH157:H7 in cow or hog manure. In fact, black soldier fly feeding on hog manure enhanced survivorship of *E. coli.* However, data from this study indicated that black soldier fly larvae significantly reduced ($F = 26775.142$, df = 1, 16, $P < 0.0001$) *E. coli* counts in dairy cow manure (Table 1). Additionally, amount of manure provided the black soldier fly larvae significantly influenced ($F = 517.491$; df = 3,16; $P < 0.0001$) the ability of the larvae to reduce *E. coli* counts. An interaction ($F = 520.683$; df = 3,16; $P < 0.0001$) between black soldier fly larvae and manure was determined.

Black soldier fly larvae significantly reduced ($F = 651.957$; df = 1,16; $P < 0.0001$) *E. coli* counts in manure as temperature increased (Table 2). Additionally, temperature significantly ($F = 137.500$; df = 1,16; $P < 0.0001$) influenced the ability of black soldier fly larvae to reduce *E. coli* counts. An interaction ($F = 165.400$; df = 3,16; $P < 0.0001$) between black soldier fly larvae and temperature was determined. Black soldier fly larvae exhibited the greatest success reducing *E. coli* counts in manure stored at 27 and 31°C. *E. coli* suppression also was substantial at 23°C (Table 2). Bacterial counts in the 35°C treatment containing larvae also indicated complete suppression in comparison to the other treatments. However, *E. coli* counts in the control at this temperature were reduced a log of 2 in comparison to controls at other temperatures. Erickson et al. (2004) also recorded a reduction of *E. coli* counts in poultry manure as temperature increased. Therefore, inactivation of the *E. coli* may not solely be caused by the black soldier fly larvae but also a result of increased temperature (El-Wadawi and Bowler 1995, Fremaux et al. 2006). This hypothesis is counter intuitive to methods implemented in this study where *E. coli* was cultured at 37°C. Therefore, *E. coli* growth was expected in the control manure at 35°C. Accordingly, Williams et al. (2006) determined that temperatures ≥50°C really are needed to destroy *E. coli* O157:H7.

We suspect the reduction in *E. coli* in the control at the greater temperature is a combination of resource quality and temperature implemented in this study not being an ideal environment for *E. coli.* Ecological and environmental condition preferences vary between *E. coli* strains. Modification of these conditions affects

Table 1. Mean *E. coli*[a] log cfu/g manure ± SD after 72 h in various amounts of dairy manure ($n = 3$) with and without (control) 125 15-d-old black soldier fly larvae and stored at 27°C, 60–70% RH, and a photoperiod of 16:8 (L:D) h in a growth chamber

Manure amount (g)	Treatments	
	Black soldier fly larvae present	Control
50	0.86 ± 0.40	8.73 ± 8.03
75	0.65 ± 2.88	8.77 ± 8.11
100	0.91 ± 3.02	9.12 ± 7.90
125	0.23 ± 3.39	8.44 ± 7.78

[a] *Escherichia coli* inoculated into dairy manure at 7.0 log cfu/g manure.

Table 2. Mean *E. coli*[a] log cfu/g manure ± SD after 72 h in 50 g dairy manure ($n = 3$) with and without (control) 125 15-d-old black soldier fly larvae and stored at 23, 27, 31, and 35°C 60–70% RH, and a photoperiod of 16:8 (L:D) h in a growth chamber

Temperature (°C)	Treatments	
	Black soldier fly larvae present	Control
23	1.86 ± 0.88	7.89 ± 6.91
27	0.90 ± 0.30	7.63 ± 6.53
31	1.99 ± 0.76	7.19 ± 6.49
35	NA	4.47 ± 4.72

[a] *Escherichia coli* inoculated into dairy manure at 7.0 log cfu/g manure.

NA, no growth.

bacterial survival and proliferation and therefore has broad consequences for the fate of these *E. coli* strains in nature (Hsiao-Hui et al. 2006). Erickson et al. (2004) also determined that manure type significantly affected survival of *E. coli* O157:H7. Different *E. coli* strains were used in this study, as well as the study of Erickson et al. (2004). Consequently, this difference in survivorship might explain why black soldier fly larvae were able to reduce bacterial counts in our study instead of their experiment.

It is not clear why larvae reared on 50 g manure at 27°C in the two experiments in this study exhibited such a considerable difference in final weight. Moisture is not suspected to have impacted larval development because of black soldier flies being able to develop in resources with moisture content ranging from 10 to 70% (Fatchurochim et al. 1989). Differences are suspected to be caused by variation in nutritional quality of the manure in combination with reduced microbial growth. Erickson et al. (2004) indicated that manure pH could affect bacteria growth. Therefore, bacteria proliferation could have been retarded if pH levels were outside an optimal range in certain manure samples. Consequently, larval weight gain would be reduced because of their potential dependence on bacteria as food.

Currently it is not known if black soldier fly larvae depend on bacteria for development. Research on other fly species that colonize manure has shown this relationship. Stable fly, *Stomoxys calcitrans* L. (Diptera: Muscidae), larvae developed on a hay and horse manure mixture containing active microbes but died when placed on sterile media (Romero et al. 2006). House fly larvae are most likely dependent on the microbial community as a nutritional source (Zurek et al. 2000). The secondary screwworm, *Cochliomyia macellaria* (F.) (Diptera: Calliphoridae), can develop better in the presence of some microbial species than others (Ahmad et al. 2006). Larvae fed blood agar inoculated with *E. coli* O157:H7 had a 35.71% survivorship, whereas those fed blood agar with *Ochrobactrum* sp. had an 81.63% survivorship (Ahmad et al. 2006). Development of black soldier fly larvae fed decomposing lean pork at 27°C (J.K.T., unpublished data) was 20–30 d longer than for larvae fed a grain diet under similar conditions (Tomberlin et al. 2002). This difference in development for black soldier fly larvae on lean pork versus a grain diet could be because of the black solder fly being selected for development on microbes specific to a resource, such as decomposing grain, rather than decomposing animal tissue and its associated microbial fauna.

Final moisture content of the manure was not determined. Therefore, it is not possible to determine whether larval feeding on the manure increased aeration of the manure and consequently reduced its moisture content and if it was a factor contributing to the reduction of bacteria and associated larval growth. However, based on bacteria counts in the controls of the first experiment (Table 1), it is apparent that bacteria remained viable as manure amount provided increased. In contrast, bacteria counts decreased in the controls as temperature increased in the second experiment (Table 2), which potentially was caused by a loss of moisture. It is important to note that, although bacteria counts in the controls did decrease with increases in temperature, they were still significantly higher than that recorded in corresponding manure samples containing black soldier fly larvae.

Table 3. Mean final total weight (g) ± SD for 15-d-old black soldier fly after 72 h in 50 g dairy manure ($n = 3$) inoculated with *E. coli* and stored at various temperatures, 60–70% RH, and a photoperiod of 16:8 (L:D) h in a growth chamber

Temperature (°C)	Mean final weight ± SD (g)
23	14.80 ± 0.30a[a]
27	12.56 ± 0.55b
31	12.73 ± 0.31b

[a] Values with different lowercase letters indicates significant different ($P < 0.05$, LSD; SPSS 2005).

Black soldier fly mortality was not measured in this study other than 100% mortality being noted for the 35°C treatment. Currently, the upper development threshold for this insect is not known. Results from this study indicate that optimal larval survivorship occurs between 23 and 31°C. Development of black soldier fly larvae on a grain diet at different temperatures indicate that they develop best between 27 and 30°C and suffered >99% mortality at 36°C (J.K.T., unpublished data). This hypothesis is further supported because of mean final larval weight (Table 3) being significantly less when reared at 27 and 31°C than 23°C.

Many blow fly (Diptera: Calliphoridae) species exhibit an upper temperature threshold during development such as that exhibited by the black soldier fly. *Chrysomya bezziana* Villeneuve (Diptera: Calliphoridae) has a similar distribution pattern throughout the temperate and tropic regions as the black soldier fly but is restricted to sub-Saharan Africa, the Indian subcontinent, Southeast Asia, and throughout the Gulf region of the Arabian Peninsula and is unable to develop when daily maximum temperatures exceeded 35°C (Siddig et al. 2005). *Calliphora vicina* (Robineau-Desvoidy) (Diptera: Calliphoridae) is a blue bottle fly encountered during the winter in the southern United States (Tomberlin and Adler 1998) and cannot survive at 35°C (Donovan et al. 2006). The black blow fly, *Phormia regina* (Meigen) (Diptera: Calliphoridae), is a forensically important species commonly encountered during the summer months in the northern United States and is dominant in the southern United States during the winter months (Byrd and Allen 2001). This species exhibited 88% mortality when reared on pork at 40°C but exhibited normal development at 35°C (Byrd and Allen 2001). They indicate 4% of resulting larvae pupated but none emerged as adults when rearing this fly with cyclic temperature range between 35 and 40°C.

Mean final weight for black soldier fly larvae generally increased with the addition of greater amounts of manure (Table 4). Larvae provided 125 g manure had the greatest weight gain in comparison to treat-

Table 4. Mean final total weight (g) ± SD for 15-d-old black soldier fly after 72 h in various amounts of dairy manure ($n = 3$) inoculated with *E. coli* and stored at 27°C, 60–70% RH, and a photoperiod of 16:8 (L:D) h in a growth chamber

Dairy manure (g)	Mean weight gain (g) ± SD
50	18.57 ± 0.38a[a]
75	23.13 ± 0.91b
100	22.22 ± 0.64b
125	25.33 ± 0.57c

[a] Values with different lowercase letters indicates significant different ($P < 0.05$, LSD; SPSS 2005).

ments provided lower amounts. These data are not surprising. Providing additional manure translates into additional resources for the larvae (Sheppard et al. 1994). However, determining the appropriate amount of resource to provide the larvae will vary based on its nutritional quality. Tomberlin et al. (2002) determined that black soldier fly larvae provided 10 g of a balanced layer hen ration with 17 ml water, which is substantially less than the amount of dairy manure provided the larvae in this experiment, resulted in greatest larval growth. Therefore, when considering the use of black soldier fly larvae as a sustainable waste management practice for a different type of waste, preliminary research is warranted to determine appropriate allocation rates.

Black soldier flies can be used to reduce livestock manure. Additionally, resulting prepupae can be used as livestock feed. Unlike in Erickson et al. (2004), the null hypothesis of this study was rejected. Data indicate that black soldier fly larvae reduce *E. coli* populations in dairy manure. However, *E. coli* were still present at low levels at the conclusion of the experiments, indicating that the black soldier fly under these experimental conditions would not be an ideal method for completely eradicating this pathogen from dairy manure. Further research is needed to determine the optimal temperature for using black soldier fly larvae as a method for reducing manure or *E. coli* populations and other potential methods for removing the remaining active bacteria. Additionally, it is not known if prepupae harvested from manure contaminated with microbial pathogens are themselves contaminated. Such research is warranted before using the prepupae as a feed for livestock. Additionally, further research is needed to determine the exact relationship between the presence of microbes and black soldier fly larval growth.

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