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Author(s): Guohui Yu, Ping Cheng, Yanhong Chen, Yongjian Li, Zihong Yang, Yuanfeng Chen, and Jeffery K. Tomberlin
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Inoculating Poultry Manure With Companion Bacteria Influences Growth and Development of Black Soldier Fly (Diptera: Stratiomyidae) Larvae

GUOHUI YU,[1] PING CHENG,[1] YANHONG CHEN,[1] YONGJIAN LI,[1] ZIHONG YANG,[1] YUANFENG CHEN,[1] AND JEFFERY K. TOMBERLIN[2]

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ABSTRACT The growth and development of black soldier fly, *Hermetia illucens* (L.), larvae fed chicken manure inoculated with bacteria isolated from black soldier fly larvae and associated larval feed was evaluated. Four strains of *Bacillus subtilis* were evaluated. *B. subtilis* strains S15, S16, S19, were isolated from the gut of black soldier fly larvae. *B. natto* strain D1 was isolated from the diet fed to black soldier fly larvae. These bacteria were added individually into nonsterile 200 g fresh hen manure at 10^6 cfu/g and homogenized. Treated manure was then inoculated with 4-d old black soldier fly larvae. Prepupal weight ranged from 0.0606 g in the control to 0.0946 g in manure treated with the S15 strain. Larval survivorship to the prepupal stage in all treatments ranged from 98.00 ± 2.65% to 99.33 ± 1.15%. Prepupal survivorship to the pupal stage ranged from 91.92 ± 1.87% to 97.95 ± 1.03%. Adult emergence from the pupal stage did not significantly ($P < 0.05$) differ across treatments and ranged from 98.95 ± 1.82% to 100.00 ± 0.00%. Adult body length resulting from the larvae in each of the treatments was significantly greater than those from the control. Longevity of adults did not differ significantly between treatments. Time from hatching to the development of the first pupa did not differ significantly across treatments; however, development time from hatching to 90% reaching the prepupual stage was significantly different between treatments and ranged from 29.00 ± 1.00 d to 34.33 ± 3.51 d. Development time from hatching to 90% reaching the adult stages was significantly different between treatments. Our results demonstrate that inoculating poultry manure with bacteria from black soldier fly larvae influences the growth and development of conspecific larvae feeding on the manure.

KEY WORDS *Hermetia illucens*, *Bacillus subtilis*, chicken manure, waste management

The black soldier fly, *Hermetia illucens* (L.) (Diptera: Stratiomyidae) is considered a beneficial arthropod (Yu et al. 2009). Its prepupae are high in protein and can be used as feed for a variety of animals, including swine (Newton et al. 1977), chickens (Hale 1973), and fish (Bondari and Sheppard 1981, 1987; St-Hilaire et al. 2007). Black soldier fly larvae can also be used to reduce manure accumulations in confined animal feeding operations (Sheppard and Newton 1994, Newton et al. 2005). However, manure management with the black soldier fly depends on a robust population for dependable inoculation of the manure with eggs. Colony methods for mass producing black soldier fly larvae have been developed (Sheppard et al. 2002). These methods depend on warm temperatures and adequate sunlight to promote mating and oviposition (Tomberlin and Sheppard 2001, 2002; Tomberlin et al. 2002; Sheppard et al. 2002).

Because of the reliance on confined animal facilities to mass produce animals for human consumption in China, there are concerns of potential environmental pollution as a result of the associated production of animal wastes in highly concentrated sites (Zhang et al. 2005, Yao et al. 2006, Li et al. 2009). Manure produced by livestock and poultry is high in nutrients (Gao et al. 2006). The average nitrogen mass and phosphorous concentration of poultry manure is 3.01 and 2.48% respectively (Yao et al. 2006). Chicken manure is ≈50% crude protein and also contains uric acid, ammonia, urea, and creatinine (Lan et al. 2001). Organic phosphorous ranges between 3.4 ≈ 13.5% (Li and Zhang 2009).

Companion microbes isolated from the black soldier fly larval gut and identified as *Bacillus subtilis* have the ability to digest protein and organic phosphorus (Yu et al. 2010a). The gut bacteria strains S15, S16m, and S19, and the bacteria *B. natto*, which was isolated from the feed provided blacks soldier fly larvae, have been shown to promote the growth and development of conspecific larvae by fermenting their food (Dong et al. 2009, Yu et al. 2010b).

[1] Zhuhai Agriculture Research Center, Zhuhai 519075, PR of China.
[2] Department of Entomology, Texas A & M University, College Station, TX 77843-2475 (e-mail: jktomberlin@ag.tamu.edu).

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The primary objective of this study was to determine basic biological parameters for the black soldier fly when reared with fresh chicken manure augmented with different companion *B. subtlis* strains individually. We compared the life-history data for prepupae, pupae, and adults for black soldier flies reared in poultry manure inoculated with different *B. subtilis* strains to data recorded for a control that was manure that did not have any of the microbes augmented. This approach was taken to determine the best companion microbe strain that increases the efficiency of chicken manure resource transformation by black soldier fly larvae. Information from this study is important for integrating the black soldier fly into current waste management strategies in poultry facilities.

Materials and Methods

Source of Flies. Black soldier fly larvae were collected from chicken manure accumulation in Zhuhai Jinding, Guangdong Province, People's Republic of China, and were reared with artificial diet (Sheppard et al. 2002) in a local colony. The flies used in the experiments represented the F_6 generation in colony following methods by Sheppard et al. (2002). Adults were managed in a 2 × 2 × 4 m screen cage held in a greenhouse (24 ≈ 30°C, 80 ± 8% RH) and watered with a misting system on the cage netting (Sheppard et al. 2002). A 12 (height) × 30 (width) × 39 (length) cm plastic basin, which contained ≈600 g saturated artificial grain-based diet (Tomberlin et al. 2002) was placed in the cage to collect soldier fly eggs. Corrugated cardboard squares measuring 6 × 3 × 1.3 cm were taped on the inside of the basin ≈3 cm above the medium. Soldier flies oviposited in the cardboard; ≈3000 eggs laid from 9:00–12:00 h on the same day were used in this study. The cardboard containing soldier fly eggs was placed in a 12 × 10 cm high cylindrical plastic container, covered with a paper towel saturated with tap water, and stored in a growth chamber (27 ≈ 30°C, 80 ± 8% RH, and a photoperiod of 14:10 [L:D] h) until larvae emerged 4 d later. Once larvae were observed, 30 g of the artificial diet mixed with 70 ml water (70% moisture) was placed in the container. The larvae were allowed to feed on the medium for 4 d before being used in the experiment. Voucher specimens are retained at the Zhuhai Agriculture Research Center, Zhuhai, China.

Source of Bacteria. *Bacillus natto* strain D1, which was supplied by Zhuhai Paierni Biological Ltd. (Zhuhai city, Guangdong Province, PR of China), can be used as feed for black soldier fly larvae (Yu et al. 2010b) and had antagonistic action toward *Escherichia coli* and *Staphylococcus aureus* (Dong et al. 2009). *B. subtilis* strains S15, S16, S19, were isolated from the gut of black soldier fly larvae collected from chicken manure, and it was determined they have the ability to digest casein, glutin, and organic phosphorous (Yu et al. 2010a). These *Bacillus* strains were inoculated in 300 ml Luria Bertani medium (LB medium) separately in 500 ml Erlenmeyer flask, and cultured at 37°C for 3 d, and then centrifuged at 10,000 × *g* to isolate the bacterial cells. The collected cells were diluted to a concentration of 10^8 cfu/ml distilled water.

Source of Chicken Manure. The chicken manure was provided by Zhuhai Breeding Bird Ltd. (Zhuhai Jinding, Guangdong Province, PR of China). The manure was produced by breeder hens which were not provided any sort of antibiotic before this study. The manure was stored at −20°C for 2 d at the Zhuhai Agriculture Research Center. The manure was allowed to thaw for 8 h before initiating the study. The defrosted nonsterile fresh chicken manure was partitioned into 200 g samples in 10 × 12 cm cylindrical plastic containers. Manure moisture content was 70.38 ± 0.91% at the initiation of the experiment.

Experimental Design. Each treatment had three replicates with each consisting of a 12 × 10 cm diaphanous cylindrical plastic container with ≈200 g of chicken manure. Each replicate of a treatment was inoculated with 2 ml of distilled water containing 10^8 cfu/ml of the assigned *Bacillus* strain. Distilled water served as the control. The manure and bacteria were homogenized. The bacteria concentration was 10^6 cfu/g manure at the initiation of the experiment. Then 100 4-d age black soldier fly larvae were added into each container, covered with cardboard breathable caps, and stored in the growth chamber previously described.

Immature Life-History Traits. Observations for prepupae were made every 24 h. Time from the initiation of the experiment to the observation of the first prepupa was recorded. Daily observations continued until all larvae had entered the prepupal stage or died. All prepupae were removed from the manure, weighed, and transferred to another 7.5 × 5.5 cm cylindrical plastic container assigned to that given treatment and corresponding replicate. Cups containing prepupae were covered with a breathable cap and placed in the growth chamber previously described, and monitored daily for the presence of pupae. The duration of prepupal development was determined. We also recorded the development time from hatch to first prepupa, and 90% pupated were recorded. We selected 90% as the final observation because of commercialization of the black soldier fly as a waste reducer and producer of feedstuff being reliant on efficiency and economic output. Reliance on 100% pupation would result in additional time and expense investment (≈7–14 d) outweighing the value of the remaining 10% larvae in the waste not recovered. Pupae were weighed and transferred to another 9 × 7 cm cylindrical plastic container. Containers were covered with a breathable cap and placed in the same growth chamber.

Adult Life-History Traits. Sex and time from hatching to adult emergence were recorded for each adult. Body length of each individual was measured with a ruler. Each individual was then placed in 2 × 8 cm glass tube covered with a cotton plug. All adults were provided 0.125 ml water daily. Tubes were placed in the same growth chamber and adult longevity recorded.

Table 1. Comparison of prepupal and pupal wt ± SE of *Hermetia illucens* reared on manure inoculated with different *Bacillus* strain treatments and control manure[a]

Bacteria treatments	Prepupal wt (g)	Pupal wt (g)
S15	0.0946 ± 0.0146a (n[b] = 274)	0.0766 ± 0.0117a (*n* = 113)
S16	0.0921 ± 0.0130b (*n* = 267)	0.0753 ± 0.0105a (*n* = 126)
S19	0.0868 ± 0.0171c (*n* = 275)	0.0739 ± 0.0133a (*n* = 80)
D1	0.0848 ± 0.0144c (*n* = 263)	0.0699 ± 0.0102b (*n* = 96)
Control	0.0775 ± 0.0122d (*n* = 228)	0.0606 ± 0.0103c (*n* = 159)

[a] Control manure was not treated with bacteria.
[b] n = replicate; means in columns followed by different letters are significantly different ($P \leq 0.05$; SPSS 2005).

Statistical Analysis. The datasets were examined to make sure they satisfied the basic assumptions of analysis of variance (ANOVA). Percent data were arcsine transformed before analysis. The data were then analyzed with the univariate ANOVA procedure (SPSS 2005). Bacterial strain served as the treatment. Fisher least significant difference (LSD) test was used following a significant *F* test ($P \leq 0.05$) to separate mean differences between different *Bacillus* strains treatments and the control for: percent survival to adult stage, prepupal weights, pupal weighs, adult male and female body length, male and female longevity, larval, prepupal, pupal development periods.

Results

Immature Life-History Traits. Mean prepupal weights of black soldier flies differed significantly for the *Bacillus* treatments and the control ($F = 53.23$; df = 4, 1290; $P = 0.001$) and ranged from 0.0775 to 0.0946 g (Table 1). Survivorship of larvae to the prepupal stage was not significantly different across *Bacillus* strain treatments and the control ($F = 0.520$; df = 4, 10; $P = 0.723$) and ranged from 95.67 to 99.33% (Table 2). Comparing with the control, the incremental percentage of prepupal weight of treatments adding *B. subtilis* strains S15, S16, S19, and *B. natto* strain D1 were 22, 19, 12, and 9%, respectively. In conclusion, at the same density of population, adding microorganisms could increase the gain of black soldier fly up to 22%.

Table 2. Percent survival ± SE of *Hermetia illucens* larvae to the prepupal and pupal stages for larvae reared on manure containing different *Bacillus* strains and control manure (n = 3)[b]

Bacteria treatments	Larval survivorship to prepupae (%)	Prepupae survivorship to pupae (%)	Adult emergence from pupae (%)
S15	99.33 ± 1.15a	96.18 ± 1.35ab	99.66 ± 0.59a
S16	95.67 ± 6.66a	97.23 ± 2.62ab	98.95 ± 1.82a
S19	98.67 ± 0.58a	97.95 ± 1.03b	99.65 ± 0.60a
D1	98.67 ± 2.31a	95.26 ± 5.54ab	100.00 ± 0.00a
Control[a]	98.00 ± 2.65a	91.92 ± 1.87a	100.00 ± 0.00a

[a] Control manure was not treated with bacteria.
[b] n = replicate; means in columns followed by different letters are significantly different ($P \leq 0.05$; SPSS 2005).

Pupal weights differed significantly among *Bacillus* treatments and the control ($F = 48.45$; df = 4, 569; $P = 0.001$) and ranged from 0.0606 to 0.0766 g (Table 1). Survivorship of prepupae was not significantly different across *Bacillus* treatments ($F = 1.89$; df = 4, 10; $P = 0.189$), but LSD test showed the survivorship of S19 treatment was significant higher than the control (Table 2).

Time from hatching to prepupal stage per treatment ranged from 22.33 to 23.00 d (Table 3) and did not differ significantly ($F = 0.583$; df = 4, 10; $P = 0.682$). Time from hatching to 90% of larvae developed to the prepupal stage per treatment ranged from 29.00 to 34.33 d and differ significantly ($F = 3.58$; df = 4, 10; $P < 0.05$) (Table 3). The duration of first to 90% larvae developed to prepupal stage per treatment ranged from 7.67 to 12.33 d and differed significantly ($F = 3.67$; df = 4, 10; $P < 0.05$).

Time from hatching to pupal stage per treatment ranged from 30.00 to 30.67 d and did not differ significantly ($F = 0.88$; df = 4, 10; $P = 0.512$) (Table 3). Time from hatching to 90% of prepupae developed to pupal stage per treatment ranged from 36.33 to 39.67 d and differed significantly ($F = 4.84$; df = 4, 10; $P < 0.05$). The duration of first to 90% percent of prepupae developed to pupal stage per treatment ranged from 7.00 to 10.33 d and differed significantly ($F = 3.38$; df = 4, 10; $P < 0.05$).

Adult Life-History Traits. Mean adult emergence rate (Table 2) did not differ significantly ($F = 0.686$; df = 4, 10; $P = 0.618$) and ranged from 98.95 to 100%. The female body length differed significantly ($F = 13.72$; df = 4, 630; $P < 0.01$) and ranged from 1.20 to 1.25 cm (Table 4). The male body length differed significantly ($F = 14.65$; df = 4, 699; $P < 0.01$) and ranged from 1.18 to 1.25 cm. Adult longevity did not significantly differ (Table 4). The female longevity ranged from 7.17 d to 7.83 d ($F = 1.38$; df = 4, 144; $P = 0.245$) and male longevity ranged from 7.60 to 8.10 d ($F = 0.801$; df = 4, 145; $P = 0.527$).

The time from hatching to adult emergence ranged from 39.33 to 40.67 d and differed significantly ($F = 4.67$; df = 4, 10; $P < 0.05$) (Table 5). The time from hatching to 90% adult emergence differed significantly ($F = 4.04$; df = 4, 10; $P < 0.05$) and ranged from 46.67 to 49.33 d. The duration of first adult emergence to 90% adult emergence ranged from 8.00 to 11.33 d and differed significantly ($F = 6.08$; df = 4, 10; $P < 0.05$). The time from hatching to all adult emergence ranged from 51.33 to 59.33 d and differed significantly ($F = 3.89$; df = 4, 10; $P < 0.05$).

Discussion

Bacteria species that have been isolated from immature flies (Ahmad et al. 2006) often are also common components of decomposing organic matter (Fitt and O'Brien 1985). Many are opportunistic pathogens (Fitt and O'Brien 1985) as in the case of *E. coli* (Liu et al. 2008) and *Samonella* (Erickson et al. 2004). However, the role played by the microbe (i.e., commensal or pathogen) is typically defined by a suite of

Table 3. Selected immature life-history traits ± SE of *Hermetia illucens* reared on manure containing different *Bacillus* strains and control manure (n = 3)[b]

Development (d)	Bacterial strain				
	S15	S16	S19	D1	Control[a]
Hatch to first prepupal stage	22.33 ± 0.58a	22.67 ± 0.58a	23.00 ± 0.00a	22.67 ± 0.58a	23.00 ± 1.00a
Hatch to 90% prepupal	29.00 ± 1.00a	29.33 ± 0.58a	32.50 ± 0.87ab	31.33 ± 2.52ab	34.33 ± 3.51b
Larva to 90% prepupal stage	7.67 ± 0.58a	7.67 ± 1.15a	10.50 ± 0.87ab	9.67 ± 2.08ab	12.33 ± 3.06b
Hatch to first pupal stage	30.33 ± 0.58a	30.00 ± 0.00a	30.67 ± 0.58a	30.67 ± 0.58a	30.33 ± 0.58a
Hatch to 90% pupal stage	36.33 ± 1.15a	38.00 ± 1.00ab	39.33 ± 0.58b	38.33 ± 1.53b	39.67 ± 0.58b
Prepupal to 90% pupal stage	7.00 ± 1.73a	9.00 ± 1.00ab	9.67 ± 0.58b	8.67 ± 1.15ab	10.33 ± 0.58b

[a] Control manure was not treated with bacteria.
[b] n = replicate; means in rows followed by different letters are significantly different ($P \leq 0.05$; SPSS 2005).

variables, such as host species (Zurek et al. 2000, Ahmad et al. 2006), corresponding health of host (Yang and Cox-Foster 2007), and environment (Liu et al. 2008). As in the case with the black soldier fly, its larvae develop in decomposing organic matter ranging from feces to grains (Sheppard et al. 1994, Erickson et al. 2004, Lu et al. 2005). Because of this selection for them to develop in such materials, there is an intimate relationship between black soldier fly larvae and bacteria associated with the resource regardless if it is human tissue (Tomberlin et al. 2005) or manure (Tomberlin et al. 2002).

In some instances, these interactions result in microbial suppression. Erickson et al. (2004) determined that black soldier fly larvae reduced *E. coli* O157:H7 6-log in chicken manure. They also determined that black soldier fly larvae had a similar effect on associated *Salmonella* populations, reducing its population by 2.5-log. Liu et al. (2008) determined black soldier fly larvae reduced *E. coli* counts in dairy manure by 6-log as well. However, it is not known if these bacteria serve as food for the larvae or the bacteria are suppressed to reduce the likelihood of infection and potential death of the larvae.

In the case of the bacteria used in this study, black soldier fly larval interactions with the targeted bacteria resulted in enhanced larval development (Table 1). Larvae fed manure augmented with the bacteria were significantly larger than those fed manure not inoculated. In comparison, stable fly *Stomoxys calcitrans* (L.) (Diptera: Muscidae) larvae fed sterile media were not able to complete development, while those provided media inoculated with bacteria species isolated from a colony of stable flies were able to successfully reach the adult stage (Lysyk et al. 1999). Similarly, Watson et al. (1993) determined house flies, *Musca domestica* L. (Diptera: Muscidae), which also develop in feces, could be raised on agar as long as it was inoculated with associated bacteria.

As in the case of this study, manure inoculated with bacteria resulted in less time being needed for black soldier flies to complete development. This result was also determined for stable flies (Lysyk et al. 1999). Gingrich (1960) determined that the absence of bacteria (i.e., sterile agar alone) required an additional 8 d to complete development. In contrast, development of the secondary screwworm, *Cochliomyia macellaria* (F.) (Diptera: Calliphoridae), was severely hampered in the presence of bacteria on sterile agar. However, unlike the black soldier fly, blow fly larvae tend to develop in decomposing carrion and not manure (Boatright and Tomberlin, 2010).

Most of the literature on the black soldier fly is restricted to its use as a biological control agent of house flies and as a manure management agent in poultry facilities. The information about the life history of this species reared in different conditions was still very rare. Our study provides additional information on the life history of this species reared on different table companion microbes treatment chicken manure. Such information is necessary for developing its potential as a waste management agent in livestock and poultry production.

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Table 4. Adult body length, emergence ratio and life-span ± SE of *Hermetia illucens* reared on manure containing different *Bacillus* strains and control manure

Bacteria treatment	Body length		Longevity	
	Female	Male	Female	Male
S15	1.25 ± 0.07c (n^b = 127)	1.25 ± 0.08c (n = 150)	7.81 ± 1.33a (n = 30)	8.10 ± 1.18a (n = 30)
S16	1.26 ± 0.08c (n = 118)	1.24 ± 0.08bc (n = 156)	7.83 ± 1.29a (n = 30)	7.77 ± 1.25a (n = 30)
S19	1.25 ± 0.08c (n = 142)	1.22 ± 0.08b (n = 136)	7.69 ± 1.51a (n = 30)	7.70 ± 1.24a (n = 30)
D1	1.22 ± 0.07b (n = 120)	1.22 ± 0.07b (n = 127)	7.40 ± 1.35a (n = 30)	7.67 ± 1.15a (n = 30)
Control[a]	1.20 ± 0.06a (n = 128	1.18 ± 0.08a (n = 135)	7.17 ± 1.51a (n = 30	7.60 ± 1.15a (n = 30)

[a] Control manure was not treated with bacteria.
[b] n = replicate; means in columns followed by different letters are significantly different ($P \leq 0.05$; SPSS 2005).

Table 5. Adult life-history ± SE of *Hermetia illucens* reared on manure containing different *Bacillus* strains and control manure (n = 3)[b]

Development (d)	Bacterial strain				
	S15	S16	S19	D1	Control[a]
Hatch to first adult emergence (d)	39.33 ± 0.58a	40.00 ± 0.00ab	40.67 ± 0.58b	40.00 ± 0.00ab	39.33 ± 0.58a
Hatch to 90% adult emergence (d)	46.67 ± 0.58a	48.00 ± 1.00ab	49.00 ± 0.00b	48.33 ± 1.53b	49.33 ± 0.58b
Pupae to 90% adult emergence (d)	8.00 ± 0.00c	8.67 ± 1.15ab	9.33 ± 0.58abc	10.33 ± 1.15bc	11.33 ± 1.15c
Hatching to all adult emergence (d)	51.33 ± 0.58a	59.33 ± 0.58b	54.67 ± 4.62a	53.67 ± 1.53a	54.67 ± 1.53a

[a] Control manure was not treated with bacteria.
[b] n = replicate; means in lines followed by different letters are significantly different ($P \leq 0.05$; SPSS 2005).

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