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Dose-dependent fate of GFP-*E. coli* in the alimentary canal of adult house flies

Kumar H.V. Naveen¹ and Dana Nayduch^{2,*}

¹ Department of Biology, Georgia Southern University, Statesboro, GA, U.S.A

² Arthropod-Borne Animal Diseases Research Unit, USDA-ARS, Manhattan, KS, U.S.A

Abstract

Adult house flies (Diptera: Muscidae; *Musca domestica* L.) can disseminate bacteria from microbe-rich substrates to areas where humans and domesticated animals reside. Because bacterial abundance fluctuates widely across substrates, flies encounter and ingest varying amounts of bacteria. We investigated the dose-dependent survival of bacteria in house flies. Flies were fed four different “doses” of GFP-expressing *Escherichia coli* (GFP *E. coli*; very low, low, medium, high, defined in text) and survival was determined at 1, 4, 10 and 22 h post-ingestion via culture and epifluorescent microscopy. Over 22 h, decline of GFP *E. coli* was significant for all treatments ($P < 0.04$) except the very low dose ($P = 0.235$). Change in survival (S) did not differ between flies fed low and very low doses of bacteria across all time points, although both treatments differed from flies fed high and medium bacterial doses at several time points. At 4, 10 and 22 h, GFP *E. coli* S significantly differed between medium and high dose-fed flies. A threshold dose, above which bacteria are detected and destroyed by house flies, may exist and likely is immune-mediated. Understanding dose-dependent bacterial survival in flies can help in predicting bacteria transmission potential.

Keywords

Bacteria; Dissemination; Vector potential

Introduction

House flies are cosmopolitan insects that breed in microbe-rich habitats because bacteria are necessary for larval development (Zurek *et al.*, 2000). Adult house flies become contaminated while visiting these sites, harboring bacteria on their body parts and disseminating them to other areas. More importantly, adult flies also ingest bacteria-rich substrates, especially those in liquid form, and move freely from these septic substrates to domestic locations, transmitting microbes in excreta droplets (West, 1951). A single adult house fly could harbor as many as 100 different potentially pathogenic bacterial species including *Escherichia coli*, *Salmonella* spp., *Pseudomonas* spp., *Streptococcus* spp., many of which cause disease in humans and domestic animals (Greenberg, 1971). As a result of this

*Corresponding author, dana.nayduch@ars.usda.gov, Tel: +1 785-537-5566, Fax: +1 785-537-5560. mnaveen1881@gmail.com

lifestyle and behavior, flies have been implicated as both mechanical and biological vectors of pathogenic microbes (Graczyk *et al.*, 2001; Zurek & Ghosh, 2014).

While bacteria harbored on the external surfaces of flies is subject to drying during flight (Yap *et al.*, 2008), several studies have shown that microbes in the alimentary canal are sequestered and have the opportunity to persist and multiply, thereby promoting biological vectoring. House flies fed *E. coli* O157:H7 harbored viable bacteria in their intestine, excreted bacteria for at least 3 d after ingestion and large numbers of bacteria were seen in the crop for up to 4 d (Kobayashi *et al.*, 1999). Flies fed *Yersinia tuberculosis* carried bacteria in their digestive tract for 36 h and disseminated bacteria for 30 h into the environment (Zurek *et al.* 2001). *Aeromonas caviae* proliferated in house flies for 2 d and persisted for up to 8 d post-ingestion, and a large number of viable bacteria were shed in vomitus and feces (Nayduch *et al.*, 2002). Similarly, *Pseudomonas aeruginosa* also proliferated and persisted in house flies, being shed in excreta for at least 24 h post-ingestion (Joyner *et al.*, 2013).

Places with vast quantities of dung or manure, such as animal rearing houses and locations without human sanitation practices, create favorable environments for the proliferation of house flies and concurrent acquisition of bacteria (Khalil *et al.*, 1994; Alam & Zurek, 2004; Meerburg *et al.*, 2007). However, the concentration, viability and species distribution of bacteria in animal dung or manure varies widely between sites and across hosts. *Salmonella* spp. have been isolated from the dung of colonized cows in concentrations ranging from 10^2 - 10^7 CFU/g (CFU=colony forming units) (Himathongkham *et al.*, 1999). Densities of *E. coli* O157:H7 in animal dung vary across individuals and host species ranging from 200-87000 CFU/g in dung of heifers (Wang *et al.*, 1996) to 10^9 CFU/g in pig feces (Kobayashi *et al.*, 2003). *Enterococcus* spp. range from 10^4 - 10^6 CFU/g in human feces to about 10^5 CFU/g from swine dung (Ahmad *et al.*, 2011). Because the concentration of bacteria shed from the host can vary widely, it follows that flies encounter and ingest highly variable amounts of bacteria during their associations with animal waste.

Assessing whether the ingested amount (“dose”) of bacteria affects survivability within and dissemination from the alimentary canal of adult house flies is important in fully understanding the risk of bacterial transmission, or vector potential. The aims of this study were to investigate the quantitative and qualitative survival of different doses of ingested GFP-expressing *Escherichia coli* (GFP *E. coli*) in the alimentary canal of flies and the subsequent transmission potential via house fly excreta.

Materials and methods

Rearing of house flies

House flies were reared in our laboratory at Georgia Southern University from a colony that was initiated in 2004. Flies were provided *ad libitum* water and fly food consisting of egg yolk, sugar, and powdered milk in a 1:2:2 ratio, respectively. The colony was maintained at 30°C with a 12 h light:12 h dark photoperiod. Larval media (3/4 wheat bran and 1/4 vermiculite) was moistened with sufficient tap water and was placed in a breeding cage to allow female oviposition. All house flies used in experiments had emerged from pupae that

were surface sanitized by washing them with freshly prepared 10 % sodium hypochlorite for 3 min. Flies were gnotobiotic for *E. coli* as determined by culture on LB agar.

Culture of green fluorescent protein (GFP) expressing *Escherichia coli*

A green fluorescent protein(GFP)-expressing strain of *Escherichia coli* DH5 α (GFP *E. coli*; Life Technologies) was used in all feeding experiments. This strain was transformed with the plasmid pGFPuv (Clontech, Mountain View, CA, USA) modified to contain an additional kanamycin resistance gene to allow dual antibiotic selection (a gift from Brian Weiss, Yale University, USA). GFP *E. coli* cultures were maintained in Luria Bertani-Ampicillin-Kanamycin agar or broth (LBAK; 25 g/l, w/v LB; 50 μ g/ml w/v each of ampicillin sodium and kanamycin sulfate) (Fisher Scientific, Atlanta, GA, USA) at 37 °C. For all fly feeding experiments, bacteria were cultured in LBAK broth to an OD₆₀₀ of 1.00-1.20 ($\pm$ 0.05), which was approximately 3.0e8 to 5.0e8 CFU/ml (CFU=colony forming units). Culture was 10-fold serially-diluted in sterile LBAK broth to obtain 4 “doses” in the feeding volume, which was 2 μ l per fly (described below in each experiment).

Preparation of flies for all feeding experiments

Newly emerged, mixed-sex flies were used for all experiments. Flies were individually-housed in sterile 30 ml glass jars and fed a 5 μ l droplet of sterile 5 % sugar water on a sterilized Parafilm® square (15 mm $\times$ 15 mm; Fisher Scientific, Atlanta, GA). Jar tops were covered with aluminum foil and flies were held at room temperature (22-25 °C) for 12 h, after which they were again provided with a sterile 5 μ l droplet of 5 % sugar water. After 12 h, flies were transferred to fresh sterile 30 ml glass jars, fasted for at 12 h at 22-25 °C and then transferred to a 30 °C incubator for 2 h to increase activity and induce feeding. During all experiments described below, flies were maintained at room temperature (22-25 °C).

GFP *E. coli* feeding, culture recovery and enumeration from adult house flies and excreta

GFP *E. coli* was cultured as described above and diluted to generate 4 doses of bacteria (mean $\pm$ SE for three biological replicates; amount shown is CFU in the 2 μ l droplet): very low, 1.05 $\pm$ 0.36e3 CFU; low, 7.27 $\pm$ 0.64e3 CFU; medium, 7.40 $\pm$ 0.53e4; high, 7.27 $\pm$ 0.31e5 CFU. Fasted flies (at least 25 per dose per replicate) were aseptically provided 2 μ l of the appropriate dose of bacteria by pipetting onto the Parafilm® square in the jar. After flies had fed, each dose was serially diluted, plated on LBAK, and incubated overnight for CFU enumeration. For enumeration of GFP *E. coli*, flies (n=5/time point) were removed at 1, 4, 10, 22 h post-ingestion (h PI). Individual flies were surface-sanitized in 70% ethanol, 10% bleach (1 min each) then air dried. Each fly was aseptically homogenized in 500 μ l of sterile LBAK broth and homogenate was serially diluted in sterile LBAK broth for duplicate culture on LBAK agar. After the flies and the Parafilm® square were removed, the jars were washed with 1 ml sterile LBAK broth to recover viable bacteria disseminated in fly excreta (i.e., fecal and vomit specks), and 100 μ l of the wash was plated on LBAK agar. All plates were incubated at 37 °C for 24 h, after which CFU of GFP *E. coli* were enumerated by standard plate count. These feeding experiments were replicated three times for each GFP *E. coli* dose. To evaluate the temporal viability of the GFP *E. coli* absent of the fly, 2 μ l of each bacteria dose (very low, 1.35e3 CFU; low, 8.20e3 CFU; medium, 7.86e4 CFU; high, 7.21e5

CFU) was added to 10 μ l sterile phosphate buffered saline (PBS, pH 7.0), held at room temperature, and similarly enumerated at 1, 4, 10 and 22 h.

Microscopic examination of GFP *E. coli* in the house fly alimentary canal

GFP *E. coli* was cultured and diluted, and individually-housed flies were provided 2 μ l of bacteria as described above: very low, 1.24e3 CFU; low, 8.00e3 CFU; medium, 7.60e4 CFU; high, 7.20 e5 CFU. At 1, 4, 10, 22 h PI, flies were immobilized at 4 °C and dissected to remove the entire alimentary canal (from proventriculus to rectum, including crop) which was microscopically examined for GFP *E. coli* (Leitz Laborlux 12 epifluorescence microscope; Wetzlar, Germany). Images were captured using a Leica DFC 420 digital camera (Leica Microsystems Ltd., Germany) and the location and cellular integrity of GFP *E. coli* was recorded.

Statistical analyses

To assess the dose-dependent survival of GFP *E. coli* in house flies, we analysed the culture-recovery data (CFU plate counts) as change in survival (ΔS), which was calculated as the difference between the number of GFP *E. coli* recovered from each fly at each time point and the number of GFP *E. coli* fed in the 2 μ l droplet. In addition, only flies that were living at each time point were included, and counts were used only if statistically valid, i.e. with plate counts between 30-300 CFU (Madigan *et al.*, 2012). Thirty-three flies out of 300 were excluded from the CFU recovery experiments from whole flies, as they did not match the above criteria; the majority of these flies (n=18) were from the group fed the very low dose. The changes in CFU (ΔS) recovered from each fly were log₁₀ transformed and checked for normality using the Shapiro-Wilk test. Subsequently, bacteria recovery over time within dose was analyzed using Kruskal-Wallis tests, and pair-wise comparisons of bacterial survival across dose and within each time interval were analysed using the Wilcoxon rank-sum test, with Bonferoni-adjusted alphas to correct for multiplicity (JMP ® 12; www.jmp.com).

Results

Temporal recovery and enumeration GFP *E. coli* from adult house flies

Flies were fed one of four doses of bacteria: very low, 1.05 $\pm$ 0.36e3 CFU; low, 7.27 $\pm$ 0.64e3 CFU; medium, 7.40 $\pm$ 0.53e4 CFU; high, 7.27 $\pm$ 0.31e5 CFU. Overall, we observed a decline (from 1 to 22 PI) in the survivability of GFP *E. coli* in house flies, irrespective of the ingested dose (Fig. 1, top panel), which was significant for all treatments ($P<0.04$) except for the very low dose ($P=0.235$). This was in contrast to results for similar doses of GFP *E. coli* in the fly-free controls (i.e., sterile PBS, Fig. 1, bottom panel), where bacteria numbers increased over time.

In the flies fed the high dose of bacteria (7.27 $\pm$ 0.31e5 CFU), 70.9 % (5.16e5 CFU) of the initial amount fed remained at 1 h PI, which amounted to a ΔS of -2.11e5 CFU (Fig. 1). There was a gradual decline in bacterial recovery throughout the collection period, with 9.8 % (7.12e4 CFU) of the fed dose remaining 22 h PI, or a total ΔS of -6.55e5 CFU. Flies fed the medium dose (7.40 $\pm$ 0.53e4 CFU) harbored 49.1 % (3.64e4 CFU) of the initial dose at 1 h PI, which was a ΔS of -3.76e4 CFU (Fig. 1). There was a sharp decrease in the

surviving bacteria at 10 h PI (6.6 %, 4.93e3 CFU) and recoverable bacteria continued to decrease until only 2.9% (2.2e3 CFU) of the ingested dose remained at 22 h PI (S , $-7.18e4$ CFU). At 1 h PI, flies fed the low dose of bacteria ($7.27 \pm 0.64e3$ CFU) still harbored 63.5 % of the amount fed (4.62e3 CFU), which was a S of $-2.65e3$ CFU (Fig. 1). Recoverable amounts of GFP *E. coli* dropped until 10 h PI, where only 28.1 % (2.04e3 CFU) remained. Interestingly, this was followed by a slight increase at 22 h PI, amounting to 33.6 % (2.44e3 CFU) of the initial dose that was fed. The total S at 22 h for the low-dose fed flies was $-4.82e3$ CFU. In flies fed the very low dose ($1.05 \pm 0.36e3$ CFU), there was an initial decline in recoverable bacteria at 1 h PI (61.8 %, 6.47e2 CFU remaining; S , $-3.99e2$ CFU), and between 1 h PI and 4 h PI (34 %, 3.62e2 CFU remaining; S , $-6.84e2$ CFU), but this was followed by an increase in bacteria in the next time interval. At 10 h PI, 67.5 % (7.06e2 CFU) of the fed dose was recovered from flies, which equated to S $-3.40e2$ CFU. A subsequent decrease in recoverable bacteria was observed at 22 h PI, where the surviving GFP *E. coli* was 61 % (6.38e2 CFU) of the initial dose and S was $-4.08e2$ CFU. However, it is important to note that 18/60 flies had to be eliminated from analysis in the very low dose-fed group because they had too few colonies to count (less than 30 CFU on the lowest dilution plate); this included 9/15 flies being excluded from the 22 h collection period. If any of these flies actually harbored no GFP *E. coli*, then the mean recoveries at 22 h PI could be significantly lower than those presented.

Statistical analysis of dose-dependent effects on GFP *E. coli* survival in adult house flies

Since our data had non-normal distribution ($P < 0.001$), a two-tailed Wilcoxon rank-sum test was used for pairwise comparisons across dose within each time point (Table 1), with Bonferroni correction ($\alpha = 0.002$). There was no significant difference in GFP *E. coli* S between flies fed the low and very low doses of bacteria across all time points, however S for each of those treatments were different from flies fed the high dose of bacteria at 4, 10 and 22 h PI. Additionally, GFP *E. coli* S in flies fed either low or very low doses of bacteria differed from flies fed the medium dose at 1, 4 and 10 h PI; however, S did not differ between the very low and medium dose-fed flies at 22 h PI, while the difference was significant for low and medium dose-fed flies at this time point. At 4, 10 and 22 h PI, GFP *E. coli* S significantly differed between the medium and high dose-fed flies.

Recovery of GFP- *E. coli* from excreta in glass jars

Excreta (vomitus and feces) were collected from the jars housing the flies in the recovery experiments above. Since most of the excreta droplets appeared to be desiccated (especially at 4, 10 and 22 h PI), recovery might not accurately reflect the true number of viable GFP *E. coli* that were initially excreted. Viable bacteria were recovered at all time points across all doses of bacteria (Table 2), except from the low dose-fed flies at 4 h PI. Within dose, the highest amount of viable bacteria was recovered at 22 h PI. The very low dose-fed flies excreted only 0.6 % (mean=6.7 CFU) of the ingested dose at 1 h PI, and the highest percentage recovered was 7.9 % (mean=83.3 CFU) at 22 h PI. Excreta from the low dose-fed flies did not carry any recoverable GFP *E. coli* at 4 h PI, but bacteria were collected at 10 h PI (1.5 % of the initial dose; mean=110 CFU). Although 1.8 % (mean=136.7 CFU) was excreted at 1 h PI, the highest percentage of the ingested dose was collected at 22 h PI (14.9 %; mean=1086.7 CFU). This reflected the highest percentage of the dose of GFP *E.*

coli excreted in any of the treatment groups. The 10 h PI recovery for the medium dose-fed flies represented the lowest percentage of bacteria (0.1 %; mean=96.7 CFU); similarly low recoveries were present at 1 and 4 h PI, with only 0.2 % (mean=176.7 CFU) and 0.3 % (mean=256.7 CFU) of the ingested dose being collected, respectively. Bacteria excretion in the high dose-fed flies was similar to that observed for the very low dose-fed flies, with a progressive increase in the number of GFP *E. coli* being collected over time. The lowest percentage of the fed dose, 0.5 % (mean=3846.7 CFU) was excreted at 1 h PI, and the CFU recovered from excreta peaked at 22 h PI, reflecting 1% of the ingested dose (mean=7630 CFU). A qualitative comparison of the temporal excretion patterns across the doses showed the low dose-fed flies dispersed the highest percentage of GFP *E. coli* in excreta, (1.8 %, 1.5 % and 14.9 %) which occurred at 1, 10 and 22 h PI, respectively. Likewise, the total percentage of the fed dose was highest for this treatment group, with a sum of 18.3% of GFP *E. coli* being excreted over the entire collection period. In contrast, at the same time points, the lowest percentages of the ingested dose were disseminated by the medium dose-fed flies, which were 0.2 %, 0.1 % and 0.6 %, respectively. Cumulatively, this equated to only 1.3% of the fed amount being excreted. Although the high dose-fed flies excreted a low percentage of their ingested dose (0.5-1 % range), that still correlated to a large number of viable GFP *E. coli* (mean=7630 CFU). In contrast to the adult fly recovery studies above, we allowed for presentation of CFU counts less than 30 for qualitative purposes, and did not perform any statistical analyses.

Microscopic examination of GFP-expressing *E. coli* in the house fly alimentary canal

In order to visually assess the fate of GFP-expressing *E. coli* and compare the spatial location of viable cells to results from culture recovery, house flies were fed four different doses of bacteria similar to the dose ranges as fed above: very low, 1.24e3 CFU; low, 8.00e3 CFU; medium, 7.60e4 CFU; high, 7.20 e5 CFU. Representative observations are shown in Figs. 2-5.

High dose—At 1 h PI, GFP-expressing *E. coli* were found in the midgut trapped and immobilized in the peritrophic matrix (PM) in flies fed with high dose of bacteria (Fig. 2A), while no bacteria were observed in the crop in any of the five flies. At 4 h PI, viable GFP *E. coli* were seen in food boluses in the posterior midgut, which started to form at this time (Fig. 2B), in all 5 flies. Similar observations were noted 10 h PI, where bacteria were enclosed in food boluses in the hindgut, and viable GFP *E. coli* cells were notably visible (Fig. 2C). At 22 h PI, food boluses were again observed in the hindgut of all 5 flies, which were noticeably smaller and contained viable GFP *E. coli* (Fig. 2D).

Medium dose—In all 5 medium dose-fed flies, at 1 h PI viable GFP *E. coli* appeared in clumps within the PM, and some bacteria were motile; however, no adherence of bacteria to the luminal surface was observed (Fig. 3A). Formation of food boluses was observed at 4 h PI, with free GFP (presumably from lysed bacteria) being observed in the gut lumen of all 5 flies (Fig. 3B). Viable cells that were present appeared to have decreased GFP expression at this time. Food boluses with free GFP were observed in the hindgut of all the 5 flies at 10 h PI and no GFP *E. coli* cells were visible (Fig. 3C). At 22 h PI, a small number of GFP *E.*

coli cells were observed in the hindgut, however they appeared to be clumped rather than in a food bolus (Fig. 3D). None of the flies harbored GFP *E. coli* cells in the crop.

Low dose—At 1 h PI, highly motile GFP-expressing *E. coli* cells were observed in the midgut region moving freely except for 1 or 2 small clumps in all 5 flies (Fig. 4A). At 4 h PI, food boluses with free GFP were prominently observed in all 5 flies, containing no discernable GFP *E. coli* cells (Fig. 4B). Three of the 5 flies at 10 h PI were observed with food boluses in the hindgut, which contained viable GFP *E. coli* cells (Fig. 4C). The remaining 2 flies had food boluses in the hindgut, but none contained any visible GFP-expressing bacteria (not shown). At 22 h PI, no viable GFP *E. coli* were observed in any of the 5 flies, and the hindgut had food boluses without free GFP (Fig. 4D); whether viable *E. coli* that were no longer expressing GFP remained in these food boluses was could not be determined. No viable GFP *E. coli* were observed in the crop of any of the flies across all time points.

Very low dose—At 1 h PI, small numbers of viable GFP-expressing *E. coli* were observed dispersed in the midgut, visibly in fewer numbers compared to other doses (Fig. 5A), and bacteria appeared in non-motile clumps in all five flies. At 4 h PI, viable bacteria were enclosed in food boluses in all flies (Fig. 5B). In all 5 flies at 10 h PI, a small amount of free GFP was observed in the food boluses and no visible GFP *E. coli* were detected (Fig. 5C). At 22 h PI, a few viable bacteria were seen in the hindgut that were not within food boluses (Fig. 5D). No viable bacteria were observed in the crop.

Discussion

The purpose of this study was to investigate whether the ingested amount (“dose”) of bacteria affected survival within the house fly alimentary canal. We utilized a GFP-expressing strain of *E. coli* to aid in visualizing and enumerating bacteria from flies, which complemented the approach used in our previous studies (McGaughey & Nayduch, 2009; Joyner *et al.*, 2013; Nayduch *et al.*, 2013; Fleming *et al.*, 2014). We measured the differential fate of bacteria as change in survival (S) since this would reveal the change in abundance from the initial amount that was ingested. Irrespective of dose, bacteria abundance in flies declined over time after ingestion. Pair-wise comparisons within each time point revealed a dose-dependent effect on this change in survival (discussed below), which is likely mediated by bacteria detection and destruction by flies. We viewed viable yet immobilized GFP *E. coli* in the alimentary canal, including some within food boluses in the hind gut. We cultured viable GFP *E. coli* from house fly excreta during our survival experiments, but this did not account for the total loss of bacteria over time. Alternatively, since we observed bacteria either immobilized by the PM, in food boluses, or even lysed in some groups, we infer that antibacterial defenses were the cause of bacteria loss in many of the flies.

There are several mechanisms by which GFP *E. coli* can be eliminated from the gut of adult house flies. Bacteria are subject to competition by other microbes in the fly gut, as has been previously shown in co-infections of flies with *Salmonella typhimurium* and *Proteus* (Greenberg, 1969), where *Proteus* out-competed *Salmonella* and caused its complete elimination by 2 d post-feeding. In a subsequent study, *Proteus mirabilis* was more

successful than *Salmonella typhimurium* in establishing within the fly midgut since it could tolerate low pH and produce antibacterial agents that inhibit other species of bacteria (Greenberg & Klowden, 1972). In our study, flies were reared gnotobiotically but not axenically; therefore, other flora were occasionally cultured in low numbers from control flies (e.g. *Pseudomonas* spp.; data not shown). However, the abundance of GFP *E. coli* outnumbered these “flora” by several orders of magnitude making the probability of out-competition unlikely.

Bacteria ingested by house flies face an onslaught of physical and chemical defenses in the alimentary canal. Adult house flies have a double-layered type II peritrophic matrix (PM) which lines the alimentary canal from midgut through hindgut, terminating at the rectum (Lehane, 1997). This barrier physically excludes bacteria from escaping the food bolus and encountering the delicate epithelial layer. We observed GFP *E. coli* trapped within the PM and most bacteria were non-motile and in food boluses beginning at 4 h PI across most flies. Immobilization may have been caused by GFP *E. coli* cells adherence to the inner luminal surface of the PM. Similar observations were previously reported by our group in flies harboring GFP-expressing *Aeromonas hydrophila* (McGaughey & Nayduch, 2009). In blow flies, lectins present on the luminal surface of the PM mediate the adherence and immobilization of bacteria. A putative binding mechanism for *Proteus vulgaris* and *Proteus morganii* was described in *Calliphora erythrocephala* (= *vicina*), where adherence to the PM luminal surface was mediated by mannose-specific lectins (Peters *et al.*, 1983). Whether the luminal surface of the house fly PM is lined with lectins remains unknown. Irrespective of the mechanism, adhered GFP *E. coli* continued to move caudally towards the rectum along with the terminal progression of the PM and peristalsis.

A synergistic mode of eliminating bacteria from the alimentary canal includes physical entrapment within the PM along with enzymatic digestion or immune-mediated destruction. pH is acidic (3.5) in the mid-midgut region (Terra *et al.*, 1988; Terra & Regel, 1995), which activates several digestive enzymes and antibacterial lysozymes. In addition to lysozyme and pH fluctuations, digestive enzymes such as proteases, carbohydrases and lipases are secreted in various midgut compartments; these enzymes diffuse into the PM and are localized for effective stability and efficient catalytic action on food digestion (Terra *et al.*, 1988; Terra & Ferreira, 1994; Lehane, 1997). However, it remains to be determined how the collective action of digestive enzymes, pH changes and/or lysozyme impact GFP *E. coli* survival in house flies.

Effector molecules of the epithelial innate immune response directly affect survival of bacteria within the gut of flies. In *Drosophila melanogaster*, the immune-deficiency (Imd) signaling cascade is induced when bacterial peptidoglycan (PGN) binds transmembrane peptidoglycan-recognition proteins (PGRPs), which activates an immune cascade resulting in the expression of bacteriocidal effectors, antimicrobial peptides (AMPs), from epithelial cells (Lemaitre & Hoffmann, 2007). AMPs interact with bacterial membranes and PGN, inhibiting proliferation or causing lysis. Interestingly, despite constant exposure to microbes in the environment, fruit flies do not constitutively express AMPs due to tightly-regulated, dose-dependent feedback inhibition. AMPs are expressed in response to Gram-negative bacteria (with *meso* DAP-type PGN) in the gut only when a normally active suppression

mechanism is overwhelmed by an abundance of dimeric PGN (Zaidman-Rémy *et al.*, 2006). PGRP-LB, which resembles membrane-bound PGRPs but instead is secreted from cells and has amidase functionality, plays the role of a scavenger, cleaving the immunostimulatory tetrapeptide off of dimeric PGN and rendering the molecule unable to bind transmembrane PGRP-LC and activate the AMP signaling cascade. Thus, circulating amidase PGRPs serve to down-regulate the immune response, where flies will not unnecessarily produce AMPs in response to low levels of bacteria (i.e., PGN) in the gut. The Imd pathway is activated when the bacterial population substantially proliferates, or is ingested over a threshold level, beyond the PGN-scavenging amidase activity of PGRP-LB in the gut lumen. In this case, those immunostimulatory fragments of PGN (dimeric moieties of DAP-PGN with intact tetrapeptide) which are not cleaved by PGRP-LB then bind transmembrane PGRPs and induce the signaling cascade and subsequent AMP production. AMPs destroy bacteria until the population reaches a level within the scavenging capacity of amidase PGRPs, completing the feedback loop where the Imd pathway is no longer stimulated. Homologs for Imd pathway components are present in the annotated house fly genome (GenBank BioProject: PRJNA210139), including PGRP-LB (GenBank Accession Number: NW_004764762). Additionally, we have previously shown that flies fed large amounts of bacteria upregulate AMP expression (Joyner *et al.*, 2013; Nayduch *et al.*, 2013; Fleming *et al.*, 2014). Therefore we presume a similar homeostatic feedback mechanism is present in the house fly gut.

Our results give insight into the dose-dependent survivability of GFP *E. coli* in flies and indicate a “threshold dose” above which a similar mechanism of PGN-Imd activation and subsequent AMP-mediated bacterial lysis occurs. Our observations indicate that this “threshold dose” would be at or above the medium dose (7.4×10^4 CFU) that was fed to flies. Pairwise comparisons of S between the very low ($\approx 1 \times 10^3$ CFU) and low ($\approx 7 \times 10^3$ CFU) dose-fed treatment groups were insignificant across all time points, indicating that the change in bacterial survival was similar irrespective of dose. However, GFP *E. coli* S in both of these treatment groups differed from that in the flies fed the medium dose ($\approx 7 \times 10^4$ CFU) and high dose ($\approx 7 \times 10^5$ CFU) at several time points. Although the numbers of bacteria in the high dose-fed flies persisted in high abundance (Fig. 1), their survival, S , declined significantly compared to the flies fed the two lowest doses of bacteria, especially at the later time points (4, 10, 22 h PI; Table 1). This may reflect the detection and destruction of bacteria by the house fly defenses in the high dose-fed flies, while the bacteria (i.e. PGN) abundance in the low and very low dose-fed flies was not “seen” by the immune response. In the flies fed the high dose of bacteria, many survived passage; we infer that although bacteria were in high enough abundance to be immunostimulatory, the sheer abundance effectively overwhelmed the limited activity of amidase PGRPs so that enough remained to be excreted.

S in both the low and very low dose-fed flies also differed significantly from the medium dose-fed flies at 1, 4 and 10 h PI. These differences, observed as soon as 1 h PI, imply early detection and destruction of significant numbers of bacteria in the medium dose treatment. At 22 h PI, S between the very low dose-fed flies and medium dose-fed flies did not differ, but this may be due to two different mechanisms of bacteria loss from flies. In the medium dose-fed flies, we observed a large amount of bacterial lysis (Fig. 3), but very little excretion (Table 2); in contrast, in the very low dose-fed flies we saw a low abundance of bacteria (Fig. 5) with little lysis, and bacteria were shed in excreta (Table 2). We infer that bacteria

abundance in very low dose flies was too low to be immunostimulatory, and likewise too few could survive in the gut and establish colonization, albeit temporary. We surmise that GFP *E. coli* in flies fed the low dose also was not above a threshold abundance to be immunostimulatory, but compared to the very low dose, was enough to establish in the gut and survive passage to the hindgut. This was supported by the observation of motile bacteria in the gut (Fig. 4), and, although some lysis was observed, these flies shed the highest percentage of the fed dose in excreta (Table 2).

In the natural environment, house flies have access to doses of bacteria on substrates such as cattle feces ranging from hundreds to millions of CFU per gram. Thus, in places with unsanitary conditions the transmission of pathogenic bacteria by the flies is quite possible but the fluctuating amount of bacteria ingested by flies will in turn make transmission risk variable. Confirmation of a “threshold dose” that stimulates AMP expression and ensuing lysis will help in determining the ultimate fate of bacteria in flies. Detailed studies incorporating similar dosages of bacteria with concurrent analysis of the local epithelial response are needed to reveal mechanisms underlying bacterial destruction in house flies.

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Figure 1. Temporal survivability of four doses of GFP-expressing *E. coli* in adult house flies and PBS controls

Top: Individual flies were fed 2 μ l of one of four different bacterial doses (CFU given in legend) and bacteria were cultured from whole house fly homogenate at 1, 4, 10 and 22 h post-ingestion (PI). Mean recoveries (Log_{10} CFU $\pm$ SEM) for each dose ($n=15/\text{time point}$) are shown. Bottom: Log_{10} CFU of GFP *E. coli* recovered from sterile 1 $\times$ PBS at 1, 4, 10 and 22 h after inoculation with 2 μ l of each bacterial dose (CFU given in legend).

Figure 2. Microscopic observation of high dose GFP *E. coli* (7.20e5 CFU) in the alimentary canal of adult house flies

(A.) Bacteria in the midgut at 1 h PI in “tracks” of inner peritrophic matrix (PM) and bacteria adhering to the lumen of the inner PM are shown (arrows). (B.) At 4 h PI, numerous viable bacteria (arrows) were enclosed in food boluses (FB), without free GFP. (C.) Food boluses in the hindgut at 10 h PI, with viable clumped bacterial cells (arrow) and free GFP. (D.) Smaller food boluses (FB) carrying viable GFP *E. coli* (arrow) were seen in the hindgut. Scale bar = 20 μm.

Figure 3. Microscopic observation of medium dose GFP *E. coli* (7.60×10^4 CFU) in the alimentary canal of adult house flies

(A.) One h PI, bacteria appeared as clumps (arrow) although they did not adhere to the PM in the midgut. (B.) 4 h PI in the anterior hindgut, clumped *E. coli* cells with low GFP expression (arrow). (C.) Large amount of free GFP was observed 10 h PI in a food bolus (FB) in the hindgut, possibly due to bacteria lysis. (D.) 22 h PI in the hindgut, GFP *E. coli* clumps (arrow). A, B, D: scale bar = 20 μm ; C, scale bar = 10 μm .

Figure 4. Microscopic observation of low dose GFP *E. coli* (8.00e3 CFU) in the alimentary canal of adult house flies

(A.) At 1 h PI, highly motile GFP-expressing *E. coli* were observed, apart from a few clumps which may have been adhered to the PM (arrow). (B.) At 4 h PI, free GFP was observed in a food bolus (FB) in the hindgut similar to the observations made in medium dose-fed flies at 10 h PI (C.) Even at 10 h PI, viable GFP *E. coli* cells were still observed in smaller boluses (arrow) in the hindgut with less free GFP. (D.) At 22 h PI, there was no free GFP observed in any of the food boluses (bright field shown). Scale bar = 20 μ m.

Figure 5. Microscopic observation of very low dose GFP *E. coli* (1.24e3 CFU) in the alimentary canal of adult house flies

(A.) 1 h PI, GFP-expressing *E. coli* in the PM lumen appeared in small clumps (arrow). (B) At 4 h PI, GFP *E. coli* (arrows) clumped in a food bolus (FB) in the midgut. (C) At 10 h PI, a small amount of free GFP was observed in some food boluses (FB) in the hindgut, with no of GFP-expressing *E. coli*. (D.) A few viable GFP *E. coli* cells were still observed 22 h PI in the hindgut (arrow) of some flies. Scale bar = 20 μ m.

Table 1

Pair-wise comparisons of the change in survival of four doses GFP *E. coli* in adult house flies.

Pair wise comparisons	1 h	4 h	10 h	22 h
Very low vs. Low	$Z-2.9234$	-3.0603	-2.9859	-2.9333
	$P0.0035$	0.0022	0.0028	0.0034
Low vs. Medium	$Z-4.295$	-3.382	-3.838	-3.532
	$P<0.0001$	0.0007	0.0001	0.0004
Medium vs. High	$Z-1.506$	-3.401	-4.188	-3.802
	$P0.1321$	0.0007	<0.0001	0.0001
Very low vs. Medium	$Z-4.188$	-3.196	-3.838	-2.933
	$P<0.0001$	0.0014	0.0001	0.0034
Low vs. High	$Z-2.025$	-3.339	-4.070	-3.802
	$P0.0429$	0.0008	<0.0001	0.0001
Very low vs. High	$Z-1.972$	-3.196	-4.070	-3.110
	$P0.0486$	0.0014	<0.0001	0.0019

Wilcoxon's rank sum test was used for pair wise comparisons of change in bacterial survival within each time point using JMP® 12. Mean CFU fed for each dose: very low, $1.05\pm0.36e3$ CFU; low, $7.27\pm0.64e3$ CFU; medium, $7.40\pm0.53e4$; high, $7.27\pm0.31e5$ CFU. Time points are hours (h) post-ingestion of given dose. Z = Z-ratio; P = Probability. Bold text indicates significant differences in pairwise comparison of change in survival after Bonferroni correction for multiple comparisons ($\alpha < 0.002$).

Table 2

Temporal recovery of viable GFP *E. coli* from the excreta of adult house flies fed different doses of bacteria.

Dose (CFU fed)	1 h	4 h	10 h	22 h
Very low ($1.05 \pm 0.36e3$)	6.7 (5.7)	10.0 (17.3)	13.3 (15.3)	83.3 (135.8)
Low ($7.27 \pm 0.64e3$)	136.7 (132.0)	0 (0) *	110.0 (190.5)	1086.7 (1686.9)
Medium ($7.40 \pm 0.53e4$)	176.7 (175.0)	256.7 (230.2)	96.7 (90.7)	493.3 (854.5)
High ($7.27 \pm 0.31e5$)	3846.7 (2564.8)	4143.3 (3804.7)	4433.3 (1851.2)	7630.0 (2669.5)

GFP *E. coli* was recovered from the excreta of house flies (n=15 per treatment and time interval) via washing glass jars in which individual flies were housed for culture recovery. Mean CFU with standard deviation in parentheses. Time points are hours (h) post-ingestion of given dose.

* Viable GFP *E. coli* colonies were not recovered from excreta.