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# A metagenomic assessment of the bacteria associated with *Lucilia sericata* and *Lucilia cuprina* (Diptera: Calliphoridae)

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1 **A metagenomic assessment of the bacteria associated with *Lucilia sericata* and**  
2 ***Lucilia cuprina* (Diptera: Calliphoridae).**

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3 **Abstract**

4 *Lucilia* Robineau-Desvoidy (*Diptera: Calliphoridae*) is a blow fly genus of forensic,  
5 medical, veterinary, and agricultural importance. This genus is also famous because of its  
6 beneficial uses in maggot debridement therapy (MDT). Although the genus is of  
7 considerable economic importance, our knowledge about microbes associated with these  
8 flies, and how these bacteria are horizontally and trans-generationally transmitted is  
9 limited. In this study, we characterized bacteria associated with different life stages of  
10 *Lucilia sericata* (Meigen) and *Lucilia cuprina* (Wiedemann) and in the salivary gland of  
11 *L. sericata* by using 16S rDNA 454-pyrosequencing. Bacteria associated with salivary  
12 gland of *L. sericata* were also characterized using light and transmission electron  
13 microscopy (TEM). Results from this study suggest that the majority of bacteria  
14 associated with these flies belong to phyla *Proteobacteria*, *Firmicutes*, and *Bacteroidetes*,  
15 and most bacteria are maintained intra-generationally, with a considerable degree of  
16 turnover from generation to generation. In both species, second generation eggs exhibited  
17 the highest bacterial phylum diversity (20% genetic distance) than other life stages. The  
18 *Lucilia* sister species shared the majority of their classified genera. Of the shared bacterial  
19 genera *Providencia*, *Ignatzschineria*, *Lactobacillus*, *Lactococcus*, *Vagococcus*,  
20 *Morganella*, and *Myroides* were present at relatively high abundances. *Lactobacillus*,  
21 *Proteus*, *Diaphorobacter*, and *Morganella* were dominant bacterial genera associated  
22 with a survey of the salivary gland of *L. sericata*. TEM analysis showed sparse  
23 distribution of both Gram-positive and Gram-negative bacteria in the salivary gland of *L.*  
24 *sericata*. There was more evidence for horizontal transmission of bacteria than there was  
25 for trans-generational inheritance. Several pathogenic genera were either amplified or  
26 reduced by the larval feeding on decomposing liver as a resource. Overall, this study  
27 provides information on bacterial communities associated with different life stages of  
28 *Lucilia*, and their horizontal and trans-generational transmission, which may help in  
29 development of better vector-borne disease management and MDT methods.

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37 **Keywords:** Microbial community, blow flies, maggot debridement therapy, salivary

1 gland, 454-sequencing.

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#### 4 **Introduction**

5 Improved biological knowledge of species from the blow fly (*Diptera*:  
6 *Calliphoridae*) genus *Lucilia* Robineau-Desvoidy, especially the sister species *L. sericata*  
7 (Meigen) and *L. cuprina* (Wiedemann), benefits basic (Singh and Wells 2013), medical  
8 (Greenberg 1973; Sherman 2009; Sherman et al. 2000; Sherman and Pechter 1988),  
9 veterinary (Stevens and Wall 1996), and forensic science endeavors (Anderson 2000;  
10 Grassberger and Reiter 2001; Sze et al. 2012; Tarone 2007; Tarone and Foran 2008;  
11 Tarone et al. 2007; Tarone et al. 2011). Since these species are primary colonizers of  
12 carrion, developmental data from these species can be useful for predicting the ages of  
13 immature blow flies associated with a body, which can help in estimating a minimum  
14 time of colonization for death investigations (Amendt et al. 2007; Tomberlin et al. 2011).  
15 They also serve as a mechanical vector of pathogens (Fischer et al. 2004; Maldonado and  
16 Centeno 2003), and are at the center of numerous neglect law suits related to the abuse of  
17 dependents, companion animals, and livestock (Hall 2005). Some species are also  
18 responsible for transmission of antibiotic resistant bacterial strains (Liu et al. 2013; Wei  
19 et al. 2014a; Zurek and Ghosh 2014).

20 Both species engage in myiasis, larval infestation of animal tissues (Ashworth and  
21 Wall 1994), which causes more than \$150 million USD of annual economic loss to the  
22 wool industry in Australia alone (Department of Agriculture and Food, Australia). This  
23 behavior has beneficial uses though, as certain *L. sericata* strains (LB-01) are useful in  
24 maggot debridement therapy (MDT) (Mumcuoglu 2001; Sherman 2009). This practice  
25 uses sterilized larvae and their preference for dead tissue to debride non-healing necrotic  
26 wounds more efficiently than a surgeon or associated treatments (van der Plas et al.  
27 2009). Given that the adults and larvae of the genus feed on feces and carrion (Clark et al.  
28 2006) and live in constant association with decomposing matter, it is not surprising that  
29 their larval excretions and secretions (ES) have been demonstrated to possess  
30 antimicrobial properties (Cazander et al. 2009a; Harris et al. 2009; Kerridge et al. 2005;  
31 Mumcuoglu et al. 2001; Sherman et al. 2000). Larval ES has also recently been

1 implicated in the ability to manipulate the development of microbial biofilms (Cazander  
2 et al. 2009b; Cazander et al. 2010; Harris et al. 2009) and to stimulate wound  
3 angiogenesis (Bexfield et al. 2010), which may explain some of their antimicrobial and  
4 bio-surgical value. Accordingly, knowledge of microbial community associated with  
5 these flies can help ameliorate the negative perception of the approach (Steenvoorde et al.  
6 2005) and promote their beneficial properties.

7         In all of the examples listed above, there is a likely microbial role that could be  
8 investigated. Insect-microbe interactions are well documented (Hilker and Meiners 2002;  
9 Ma et al. 2012b; Schröder and Hilker 2008). Microbial communities can affect life-  
10 history traits (Ma et al. 2012a), and sex ratios (Hurst and Jiggins 2000), which can both  
11 influence the survival of a population. Microbes can also influence attraction of insects to  
12 their hosts (Hilker and Meiners 2002). For instance, *Proteus mirabilis* attracts *L. sericata*  
13 (Ma et al. 2012b; Tomberlin et al. 2012); *Musca domestica* Linnaeus (*Diptera: Muscidae*)  
14 females have been shown to prefer to oviposit on eggs coated with certain Gram-positive  
15 bacteria (Lam et al. 2007), and *Aedes aegypti* (Linnaeus) (*Diptera: Culicidae*) prefer  
16 oviposition on mixture of 14 bacteria isolates from bamboo leaf-infusion compared to  
17 water as a control (Ponnusamy et al. 2008). Since bacteria and their associated  
18 metabolites can influence blow fly behavior, it seems likely that bacterial research with  
19 these flies will have repercussions for forensic, medical, veterinary, and agricultural  
20 applications (Tomberlin et al. 2012).

21         Identifying the potential microbial contaminants of experiments is important for  
22 deciphering the variation observed in research observed with these species. While maggot  
23 debridement therapy has been shown to decrease the prevalence of some microbes on a  
24 wound, other microbes are unaffected or increase in prevalence in association with  
25 treatment with *L. sericata* larvae (Jaklic et al. 2008). Sterile techniques for rearing *Lucilia*  
26 *sericata* are well established (Mumcuoglu et al. 2001; Sherman and Tran 1994) but in  
27 some situations (e.g., use of non-sterile maggots instead of sterile maggots) MDT can  
28 also cause septicemia (Mumcuoglu 2001). In some situations more than two species can  
29 colonize a wound pre-mortem, which can complicate calculation of minimum  
30 postmortem interval estimation when using insect evidence in death investigations  
31 (Sanford et al. 2014). In all of these cases, knowledge of microbes associated with non-

1 sterile larvae would aid in 1) identifying the likely sources of septicemia in the case of  
2 failed maggot debridement therapy, 2) interpreting the results of potentially non-sterile  
3 ES experiments, 3) identifying bacteria that are unaffected by the feeding of *Lucilia*  
4 larvae, and 4) identify bacteria that attract different blow flies for oviposition pre- or post-  
5 mortem.

6         These considerations raise several questions regarding potential bacterial  
7 communities associated with these important blow flies: 1) What bacteria are associated  
8 with these species, and how similar are the bacterial communities associated with each  
9 species?; 2) What bacteria are likely to be trans-generationally transmitted and what  
10 bacteria are likely to be horizontally transmitted?; and 3) What bacteria are amplified or  
11 eliminated by larval feeding? To address these questions, we conducted a survey of  
12 bacterial communities associated with these sister species using 16S rDNA 454-  
13 pyrosequencing.

## 14 **Materials and Methods**

### 15 **Fly colony maintenance**

16         *Lucilia sericata* were collected from Davis, CA, USA in 2006 and maintained as  
17 previously described (Tarone and Foran 2008). The transcriptome of this strain is  
18 published (Sze et al. 2012). *Lucilia cuprina* were collected from the “Miracle Mile”  
19 neighborhood and University of Southern California campus in Los Angeles, CA, USA in  
20 2007 (Li et al. 2014) and maintained in the same conditions as *L. sericata*. Both species  
21 were identified by both morphological and molecular methods using identification keys  
22 as previously described (Tarone and Foran 2006; Tarone and Foran 2008; Whitworth  
23 2006).

### 24 **Sample collection**

#### 25 *Fly life stages*

26         Generationally related eggs, larvae, pupae and adults (male and female) were  
27 raised in the same environment on raw beef liver. Each of the experiments was done with  
28 one replicate per species, as the goal was to 1) categorize bacteria associated with the  
29 flies and 2) determine if it appeared likely that bacteria were mostly horizontally or trans-  
30 generationally inherited. Approximately 0.5 g eggs (1<sup>st</sup> generation eggs or G1egg) were  
31 removed for DNA extraction. The remainder of the eggs was left to hatch and was

1 harvested sequentially as the flies developed. The resulting 3<sup>rd</sup> instar larvae (Larva),  
2 pupae (Pupa), adult males (AM), adult females (AF), and 2<sup>nd</sup> generation eggs (G2egg)  
3 were randomly collected and frozen at -80°C until DNA extraction could be performed.

#### 4 *Salivary gland removal protocol*

5 Because *L. sericata* larvae exhibit special salivary gland chemistry important in  
6 maggot debridement therapy, we also surveyed bacteria associated with the salivary gland  
7 of *L. sericata* third instar larva. *L. sericata* from a separate cohort was raised at room  
8 temperature on beef liver. Feeding third instars with full crops were collected with  
9 forceps and transferred in a non-sterile plastic cup to the dissection area. Maggots were  
10 washed in a 1.25% sodium hypochlorite solution followed by two washes in sterile  
11 phosphate buffered saline (PBS). Salivary glands were dissected with sterile forceps  
12 under a stereomicroscope and placed in sterile PBS on ice. This process was repeated  
13 thrice to obtain a concentration of one salivary gland per 10mL of PBS (one pair of  
14 salivary glands per 20mL) was achieved. The extracted salivary glands were either  
15 collected for transmission electron microscopy (TEM) or homogenized with a sterile  
16 Teflon pestle and were used for DNA extraction and 454-pyrosequencing. For the TEM  
17 experiment, crops from the same individuals were also collected and analyzed as a  
18 positive control for the presence of bacteria.

#### 19 *Transmission electron microscopy*

20 Salivary glands were preserved in a fixative consisting of 3% glutaraldehyde, 2%  
21 paraformaldehyde and 12% picric acid prepared in 50 mM phosphate buffer, pH 7.4, and  
22 50 mM sucrose. Salivary glands in fixative were incubated at room temperature for 60  
23 min. then held at 4°C. Subsequent to primary fixation, salivary glands were postfixed for  
24 2 hr. at 4°C in 1% osmium tetroxide prepared in 100 mM phosphate buffer, pH 7.4,  
25 100mM sucrose and 50 mM K<sub>4</sub>Fe(CN)<sub>6</sub> (potassium ferricyanide). After osmication,  
26 samples were rinsed at 4°C in 50 mM phosphate buffer, pH 7.4, containing 50 mM  
27 sucrose followed by eight rinses in 4°C distilled H<sub>2</sub>O over the course of 2 hr, then post-  
28 staining overnight at 4°C in 0.5% uranyl acetate. Following post-staining, samples were  
29 rinsed in 4°C distilled H<sub>2</sub>O and dehydrated in a graded ethanol series, and acetone.  
30 Dehydration was followed by infiltration and embedding in Mollenhauer's formulation of  
31 epoxy resin (Mollenhauer 1964). Thin TEM sections, 70 nm, were cut and stained using



1 1% uranyl acetate and lead citrate then viewed in a Hitachi H7000 transmission electron  
2 microscope. Sections 750 nm, for light microscopy were stained with either 0.05%  
3 toluidine blue or a mixture of basic fuchsin and toluidine blue (Multiple Stain,  
4 Polysciences, Warrington, PA, USA).

5 *Determining the proportion of bacteria that are horizontally and trans-generationally*  
6 *inherited*

7 To better understand the dynamics of bacterial exchange between the environment  
8 and *L. sericata*, an experiment was conducted to allow adult flies to oviposit on three  
9 different commercial sources of liver (previously frozen at -20°C) and follow the flies  
10 that developed (Fig. S1). The bacteria from the adults and liver prior to oviposition and  
11 from 3<sup>rd</sup> instars and the liver after development were evaluated. The three liver sources  
12 were collected from different supply chains (x, y, and z) to maximize the variation in  
13 liver-associated microbes. Four 0.25 g replicate samples were randomly collected from  
14 each liver sample prior to exposure to adult flies (fresh liver) and after use by and  
15 removal of flies (aged liver). Four replicate samples each of 6 (3 male and 3 female) adult  
16 flies prior to access to the liver (adult) and of 0.25 g 3<sup>rd</sup> instar larvae that were oviposited  
17 and had grown on the specific liver sources (larvae) were randomly collected. Samples  
18 were stored at -80°C until DNA extraction was performed. The experiment was replicated  
19 three times.

20 **DNA extraction**

21 DNA extractions were performed from 0.25 g liver tissue, 0.25 g eggs (1 hour  
22 old), two larvae (7-day old), two pupae, and two newly emerged adults. These samples  
23 were selected randomly and whole insect specimens were homogenized in 1.5 ml PBS.  
24 Briefly, homogenized samples were placed in 1.5 ml microcentrifuge tubes with 500 µl  
25 Tris-EDTA (pH=8), 50 µl 10% SDS, 3 µl proteinase K (20 mg/ml), 1.5 µl of lysozyme  
26 (50 mg/ml) and then incubated with shaking (900 rpm) at 56°C in a water bath. After 1  
27 hour of incubation, 100 µl NaCl (5M) and 80 µl CTAB extraction solution (Teknova,  
28 USA) were added and samples thoroughly mixed and incubated at 65°C for 10 minutes.  
29 Sequential extraction in a 1X volume was performed using phenol (pH 8.0),  
30 phenol/chloroform/isoamyl alcohol (25:24:1), and chloroform/isoamyl alcohol (24:1) by  
31 centrifugation at 6000x g for 6 minutes. The DNA was precipitated in 0.7 volume of

1 isopropanol, washed twice in 70% ethanol, dissolved in nuclease free water, and  
2 quantified by spectrophotometry. Extracted DNA was aliquotted and sent to Research and  
3 Testing Laboratory (<http://www.researchandtesting.com/>) for 16S rDNA 454-  
4 pyrosequencing using universal bacterial primer pair 27F (5'-  
5 GAGTTTGATCNTGGCTCAG) and 519R (5'- GTNTTACNGCGGCKGCTG) by  
6 bacterial tag-encoded FLX-Titanium pyrosequencing (bTEFAP) method (Dowd et al.  
7 2008) in Genome Sequencer FLX System (Roche, Nutley, NJ, USA). All FLX related  
8 procedures were performed following Genome Sequencer FLX System manufacturers  
9 instructions (Roche, Nutley, NJ, USA).

### 10 **Pyrosequencing data analysis**

11 Sequences with lengths less than 150 bp were removed and remaining sequences  
12 (103629) were checked for chimera formation using web based chimera check program  
13 Decipher (Wright et al. 2012) (<http://decipher.cee.wisc.edu/FindChimeras.html>) (accessed  
14 on April 19, 2012). Suspected chimeric sequences (6461) were deleted from the dataset  
15 and only chimera free sequences (97168) were used for further analyses. Hierarchical  
16 classification of the 97168 16S rDNA sequences were carried out according to the  
17 Bergey's bacterial taxonomy (Garrity et al. 2004) using Naïve Bayesian rRNA classifier  
18 version 2.2 (Wang, *et al.*, 2007) as implemented in Ribosomal Database Project (RDP)  
19 Multiclassifier version 1.0. Only sequences having  $\geq 80\%$  bootstrap support were  
20 considered classified at a particular hierarchical level. Venn diagram of all classified  
21 sequences were created using software Vennture (Martin et al. 2012)

22 Heat map graphics were generated by using gplots package in R version 2.13.0 (R  
23 Development Core Team 2006) for all genera that were present at  $\geq 0.5$  percent relative  
24 sequence abundance. For better visualization % relative sequence abundance values were  
25 natural log transformed before its use in the heat map. The 0% values were converted into  
26 0.01% for log transformation. Bacterial genera were clustered based on rooted NJ tree (Y-  
27 axis) (See below for detail) whereas fly life stages and bacterial sources were clustered  
28 based on FastUniFrac based clustering (X-axis) which helps in better comparison of  
29 bacteria by phenotypic and taxonomic characteristics important to bacterial community  
30 functional analysis.

31 Duplicate and nearly duplicate sequence from each data set (*L. sericata* including

1 salivary gland data, *L. cuprina* and bacterial sources) were removed using default  
2 parameters in CD-HIT 454 (Niu et al. 2010), and only unique sequences (<98% sequence  
3 similarity) from each data sets were used for the construction of neighbor-joining (NJ)  
4 trees. NJ trees were rooted based on 16S rRNA gene sequence of *Thermatoga maritima*  
5 (M21774) and *Aquifex pyrophilus* (M83548). For NJ tree construction all data sets were  
6 aligned based on 16S rRNA secondary structure in Infernal aligner (Nawrocki and Eddy  
7 2007; Nawrocki et al. 2009), as implemented in the Ribosomal Database Project (RDP)  
8 under tool Aligner (<http://rdp.cme.msu.edu/>) (accessed on October 22, 2012).  
9 Hypervariable ambiguous regions were manually deleted from the multiple sequence  
10 alignment in MEGA5 (Tamura et al. 2011). Evolutionary distances of aligned sequences  
11 were calculated by NJ method with the Kimura two-parameter correction (Saitou and Nei  
12 1987) for 1000 bootstrap replications in PAUP\* v.4.0b10 (Swofford 2003). Calculated  
13 evolutionary distances were used for construction of rooted NJ trees in PAUP\* v.4.0b10  
14 (Swofford 2003).

15         Approximate maximum-likelihood trees were constructed from all sequences  
16 (including outgroups *Thermatoga maritima* (M21774) and *Aquifex pyrophilus* (M83548)  
17 16S rDNA sequences) of each data set using default parameters in FastTree2 (Price et al.  
18 2010). Approximate ML trees were used as an input file in FastUniFrac based clustering  
19 of bacterial communities (Hamady et al. 2009) associated with different samples.  
20 Jackknifing with 1000 permutations was performed for node support of the FastUniFrac  
21 tree. *P*-tests were performed using 1000 permutations for each pair of samples and for all  
22 samples together in FastUniFrac (Hamady et al. 2009). All trees were edited using  
23 Archaeoptryx version 0.957 beta (Han and Zmasek 2009) and FigTree v1.3.1  
24 (<http://tree.bio.ed.ac.uk/>).

25         Diversity indices were calculated using tools available in RDP pyrosequencing  
26 pipeline (<http://pyro.cme.msu.edu/>). Rarefaction curves were generated in Excel 2007  
27 (Microsoft Corporation, Redmond, WA) using results obtained from the tools aligner,  
28 complete linkage clustering, and rarefaction of RDP pyrosequencing pipeline (Cole et al.  
29 2009) (<http://pyro.cme.msu.edu/>; accessed on October 23, 2012). Shannon (1948) and  
30 Chao1 (2002) indices were calculated using tool Shannon and Chao1 index of RDP  
31 pyrosequencing pipeline (Cole et al. 2009) (<http://pyro.cme.msu.edu/>; accessed on

1 October 23, 2012). Percentage coverage of species richness was calculated from  
2 rarefaction and Chao1 indices using method as described in Zheng et al. (2013). All raw  
3 sequence files were submitted to Sequence Read Archive (SRA). Study accession #  
4 PRJEB6623 can be used for the retrieval of raw sequences used in this study.

## 5 **Results**

### 6 **General characteristics of 454-sequences**

7 This study produced 29792 chimera free bacterial sequences with an average  
8 length of 296 bp. These samples came from successive life stages of the blow fly sister  
9 species *L. cuprina* and *L. sericata*. The number of sequences obtained from first  
10 generation eggs (G1egg), larvae, pupae, male adults (AM), female adults (AF) and  
11 second generation eggs (G2egg) samples were 1965, 1961, 3081, 2415, 4451, 234 in *L.*  
12 *cuprina* and 3053, 4113, 1752, 2583, 3896, 288 in *L. sericata*, respectively. In *L. cuprina*,  
13 approximately 99.7%, 98.8%, 98.1%, 92.7%, and 82% of all sequences were classified  
14 with  $\geq 80.0\%$  bootstrap support into 5 phyla, 11 classes, 17 orders, 42 families, and 59  
15 genera, respectively. On the other hand, in *L. sericata* approximately 99.9%, 99.7%,  
16 99.4%, 98.2 and 76.5 % of all sequences were classified with  $\geq 80.0\%$  bootstrap support  
17 into 7 phyla, 13 classes, 22 orders, 49 families, and 83 genera, respectively. Additionally  
18 1283, 13347, 22790, 17261, and 12695 sequences were also obtained from *L. sericata*  
19 salivary gland, *L. sericata* adults, *L. sericata* third instar larvae, fresh liver, and aged liver  
20 respectively (see Fig. S1 for experimental design). In these samples, approximately  
21 99.6%, 99.5%, 97.9%, 94.8%, and 77.0% of all sequences (respectively) were classified  
22 with  $\geq 80.0\%$  bootstrap support into 6 phyla, 11 classes, 20 orders, 38 families, and 47  
23 genera, respectively.

### 24 **Taxonomic distribution of 454-sequences**

25 The majority of sequences ( $>99\%$ ) collected from successive life stages of *Lucilia*  
26 belonged to the phyla *Proteobacteria*, *Firmicutes*, and *Bacteroidetes* (Fig. 1; Table S1).  
27 Phylum level relative sequence abundances associated with male and female adult *L.*  
28 *sericata* flies were almost the same (mainly *Proteobacteria*), but this was not true with *L.*  
29 *cuprina* male and female adults (Fig. 1; Table S1). *Acidobacteria* and *Actinobacteria*  
30 were mainly associated with second-generation eggs (G2egg) in both species.  
31 *Fusobacteria* was mainly present in *L. sericata* second-generation eggs (G2egg) samples.

1 Similarly, more than 90% of all classified sequences across all life stages belong to the  
2 classes *Gammaproteobacteria*, *Bacilli*, and *Flavobacteria* and orders *Enterobacteriales*,  
3 *Xanthomonadales*, and *Lactobacillales* in both *Lucilia* species (Table S1). Additionally,  
4 *Flavobacteriales* and *Bacillales* were present at relatively higher sequence abundances in  
5 pupal samples of both *Lucilia* species. At the family level, *Enterobacteriaceae*,  
6 *Xanthomonadaceae*, *Lactobacillaceae*, and *Enterococcaceae* were present in high  
7 numbers across all life stages of *Lucilia* spp. (Table S1). *Flavobacteriaceae* were mainly  
8 present in the pupal stage of both *Lucilia* species (Table S1). Although the blow fly sister  
9 species shared the majority of their classified genera (42 genera), there were some that  
10 were only observed in one species (Fig. 2). Of the bacterial genera identified  
11 *Lactobacillus* (25%), *Providencia* (24%), *Ignatzschineria* (10%), *Lactococcus* (8%) and  
12 *Vagococcus* (4.4%) were the five most dominant genera associated with *L. cuprina*,  
13 whereas *Providencia* (53%), *Ignatzschineria* (5%), *Myroides* (4%), *Lactobacillus* (3%),  
14 and *Morganella* (2.6%) were the five most dominant genera associated with *L. sericata*  
15 (Fig. 3; Table S1). Pupae of both blow fly species had relatively high abundances of  
16 *Myroides*.

17 In both species, a FastUniFrac based *P*-test suggests that bacterial communities  
18 differ significantly between life stages ( $p \leq 0.001$ ) and bacterial communities associated  
19 with each of the life stages are significantly clustered ( $p \leq 0.001$ ). An unweighted  
20 FastUniFrac based tree, which is based on composition (and not quantity) of bacteria  
21 associated with each sample, shows similar clustering pattern between life stages in both  
22 blow fly species. In both species the adult female shares more bacterial taxa with G1egg,  
23 than to either the adult male or any other life stages. Similarly, the larval stage shares  
24 more bacterial taxa with pupae, than to any other life stages. In both species, the G2egg  
25 stage shared the least number of bacteria with other life stages (Fig. 3) and yielded the  
26 least numbers of sequences. Relationships between different life stage samples were not  
27 the same in *L. sericata* and *L. cuprina* in a weighted FastUniFrac based tree (Fig. S2a &  
28 b).

### 29 **Bacterial richness and diversity indices**

30 In *L. cuprina*, bacterial diversity at species (3% sequence divergence) and genus  
31 (5% sequence divergence) levels was similar in all life stages, but at the phylum level

1 (20% genetic divergence), diversity was relatively higher in G2egg than any other life  
2 stages (Table 1). In *L. sericata*, at species (3% sequence divergence) and genus (5%  
3 sequence divergence) levels, bacterial diversity was almost same in all life stages, except  
4 in male adult samples, where bacterial diversity was lowest compared to all other life  
5 stage samples at all sequence divergences. At 20% sequence divergence, bacterial  
6 diversity was relatively higher in G2egg and pupal samples. Similar trends were observed  
7 with rarefaction and Chao1 estimators (Table 1, Fig. S3). Sequencing effort covered more  
8 than 60% of bacterial diversity at species level (except *L. sericata* pupa), more than 66%  
9 at genus level (except G2egg in *L. cuprina*, and pupal samples in *L. sericata*), and more  
10 than 80 % at phylum level (except G1egg and pupa in *L. cuprina*).

### 11 **Bacteria in the salivary glands of *L. sericata***

12 Bacteria in the salivary glands of *L. sericata* were assessed using two different  
13 techniques: pyrosequencing and microscopy. Based on sequencing results, the two most  
14 dominant phyla, classes, orders, and families associated with the *L. sericata* salivary  
15 gland were *Firmicutes* (52.1%) and *Proteobacteria* (41.9%), *Bacilli* (44.1%) and  
16 *Gammaproteobacteria* (28.7%), *Lactobacillales* (41.5%) and *Enterobacteriales* (27.1%),  
17 and *Enterobacteriaceae* (27.1) and *Lactobacillaceae* (22.0%), respectively. The salivary  
18 gland community structure was more similar to G2egg than to any other life stages of *L.*  
19 *sericata* (p-value <0.001) (Fig. 3b). Among classified bacterial genera, more than 60% of  
20 the sequences belonged to the genera *Lactobacillus*, *Proteus*, *Diaphorobacter*, and  
21 *Morganella* in decreasing order in the salivary gland of *L. sericata* (Fig. 4). The salivary  
22 glands were also evaluated by TEM, using a comparison to crops (Fig. 5). Crops were  
23 full of bacterial cells, yielding an array of bacterial cell types throughout. In contrast,  
24 bacterial cells were sparse in the salivary glands. Only a few bacterial cells were found in  
25 the salivary gland after evaluation of numerous slices from 20 maggots, but this is  
26 partially due to the delicate structure of the gland making sectioning a challenge.  
27 Structures indicative of both Gram-positive and Gram-negative cells were located within  
28 the salivary duct, supporting the sequencing observations (Fig. 5).

### 29 **Trans-generationally and horizontally transmitted bacteria**

30 Bacterial communities associated with fresh liver and aged liver samples were  
31 more similar to each other than to either *L. sericata* adults that landed, ate, and oviposited

1 on the liver or the *L. sericata* larvae that had fed upon the liver in both weighted and  
2 unweighted FastUniFrac based clustering (Fig. S4). Adult and aged liver samples shared  
3 12 bacterial genera that were not present in larval and fresh liver samples. On the other  
4 hand, *L. sericata* adult and larval samples did not share any bacteria that were not present  
5 in other samples. Total 15 genera were shared by all samples (adult, larva, fresh liver, and  
6 aged liver). Out of 15 genera, *Proteus*, *Enterococcus*, and *Lactobacillus* were the  
7 dominant genera that were present in all samples (Fig. 6a & 6b). Several pathogenic  
8 genera were also present in adult and/or fresh liver samples, which either got amplified or  
9 reduced by larval activities (Fig. 7).

## 10 **Discussion**

11 This study was designed to evaluate the bacterial communities associated with  
12 two sister *Lucilia* species (*L. sericata* and *L. cuprina*), which are important to medicine,  
13 agriculture, veterinary, and forensic science. The work was designed to ask which  
14 bacteria are associated with each species and how similar are their respective bacterial  
15 communities, which bacteria are horizontally or trans-generationally transmitted, and  
16 which are amplified or eliminated during larval feeding

17 The first part of the study evaluated an un-replicated (at the level of fly species)  
18 developmental time series of fly-associated bacterial communities, starting with eggs,  
19 proceeding throughout development, and culminating in a second generation of eggs.  
20 These data are useful for establishing the presence of certain members of the bacterial  
21 communities, but absence and concentration information should be carefully considered  
22 with the fact that replication was not done per time point per species. With this caveat in  
23 mind, it is interesting to note that many of the same bacteria appeared in both time series,  
24 it was clear that there was a different community composition associated with species,  
25 representing numerous taxa, mostly from those phyla found in the human (Backhed et al.  
26 2005) and insect (Gupta et al. 2012; Gupta et al. 2014; Wei et al. 2014b; Zheng et al.  
27 2013) gut. Relative abundances appeared to differ between species, but this portion of the  
28 study was not replicated within species, making it impossible to differentiate replicate  
29 effects from species effects. Given that limitation, both time series observations still  
30 demonstrated that each sister species of *Lucilia* consists of some putatively unique and  
31 many shared bacterial genera, with a large turnover in community occurring for both

1 species at oviposition.

2         Among shared bacterial genera, *Providencia* and *Ignatzschineria* were present in  
3 relatively high abundance in the sister species of *Lucilia*. These genera were also  
4 observed with several other carrion-breeding flies (Gupta et al. 2012; Gupta et al. 2014;  
5 Wei et al. 2014b; Zheng et al. 2013), and hence it looks like they are typical bacterial  
6 genera of carrion breeding flies. *Providencia* produces several Xylanases, and helps in  
7 decomposition of xylan, which is commonly observed at decomposition sites (Raj et al.  
8 2013). *Ignatzschineria* is strong in chitinase activity, and its high abundances in larval  
9 and pupal samples suggest that it may be playing a significant role in insect  
10 metamorphosis (Toth et al. 2001). Although *Lactobacillus* was shared by both *Lucilia*  
11 species, its relative abundance was comparatively higher in *L. cuprina* than in *L. sericata*.  
12 *Lactobacillus* is also commonly observed at decomposition sites, and is known to inhibit  
13 growth of many harmful bacteria by making environment acidic. Similarly, *Myroides*  
14 (*Flavobacteriaceae*) was present at comparatively high abundance in pupal samples,  
15 which most probably protect pupa from harmful environmental bacteria, because  
16 *Myroides* produces bio-surfactants with known antibacterial properties (Dharne et al.  
17 2008; Spitteller et al. 2000).

18         At the commencement of a new generation, bacterial communities associated with  
19 eggs were considerably altered from the previous generation, even from that of the  
20 maternal bacterial communities. Trans-generationally inherited bacteria in G2egg might  
21 have come either from the mother or from environment. In both *Lucilia* species, G2egg  
22 samples differed from other life stages mainly because of relatively high abundance of  
23 *Acidobacteria* and *Actinobacteria* (Fig. 1). The genome of *Acidobacteria* contains several  
24 cellulose and protein synthesizing genes (Ward et al. 2009). A network of bacterial  
25 celluloses can produce biofilm, retain water under dry conditions, and helps in aeration.  
26 All these functions of the network of celluloses most probably contribute in egg structure  
27 and protection of eggs from desiccation (Ward et al. 2009). Members of *Actinobacteria*  
28 are known to produce several antimicrobial bioactive compounds, which may be  
29 protecting egg from harmful bacteria and fungi (Mahajan and Balachandran 2012;  
30 Raghava Rao et al. 2012). This may also be a reason why we see relatively less bacterial  
31 sequences in egg samples compared to other life stage samples. This was seen previously



1 in the black soldier fly, *Hermetia illucens* (L.) (*Diptera: Stratiomyidae*) (Zheng et al.  
2 2013), and it remains to be seen if this is a property of the experimental design or a  
3 feature of carrion fly biology. *Fusobacteria*, which is a causative agent for bacteremia,  
4 was observed only in the G2egg of *L. sericata*, which suggests to us that these bacteria  
5 may be the responsible agent for the fatal myiasis, sometimes caused by *L. sericata*  
6 (Henry et al. 1983; Mowlavi et al. 2011).

7         The results of these initial observations would indicate that many of the bacteria  
8 associated with carrion flies are acquired from the environment. This has implications for  
9 the management of pathogen transmitted by these insects and could explain a proportion  
10 of the variation measured in the development of these flies on different resources. It  
11 should also be noted that, within a generation, many of the same taxa were observed at  
12 multiple life stages, suggesting that replication of experiments is more important between  
13 generations than within. This also suggests that, once oviposition has occurred, larvae  
14 (and subsequent) life history stages retain many of the microbes in their community.  
15 Thus, there may be high selective pressures on maternal choice of potential larval  
16 resources driven by the bacteria present, particularly if any of those bacteria have fitness  
17 effects on flies. This also indicates a need for larval plasticity with respect to adapting to  
18 the variation in bacterial community structure on larval resources, since even  
19 communities found on the same resource type may vary considerably.

20         To specifically address whether bacterial communities were trans-generationally  
21 or horizontally inherited, a set of replicated observations were made using *L. sericata*.  
22 Three different groups of adults were presented with three different liver sources and  
23 allowed to lay eggs on them. These flies, their oviposition substrate, their offspring, and  
24 the substrate after growth of the offspring on the substrate were all evaluated using  
25 metagenomic approaches. Several observations were made from the results as shown in  
26 the Venn diagram and heat maps (Figs. S4, 6a and 6b).

27         First, in unweighted FastUniFrac clustering, the bacterial community structures  
28 associated with *L. sericata* adults was more similar to fresh and aged liver samples than  
29 to larval sample, whereas in weighted FastUniFrac clustering, bacterial community  
30 structures associated with fresh and aged liver samples was more similar to larval  
31 samples than to adult samples (Fig. S4). Because weighted FastUniFrac clustering is

1 based both on bacterial composition and quantity (compared to just bacterial composition  
2 in unweighted FastUniFrac clustering), a close relationship between liver and larval  
3 samples in weighted FastUniFrac clustering is most probably because of similar numbers  
4 and types of taxa in these samples, suggesting convergence in communities due either to  
5 larval manipulation of the bacterial community on the liver or the ability of larvae to  
6 persist in the community found on the liver without needing to regulate its own  
7 community. For example, *Vagococcus* and *Lactobacillus* were present at very high %  
8 relative abundances (>25%) in larval and liver samples, but their relative abundances  
9 were significantly low (<1%) in adult samples.

10 Many bacterial genera are common throughout the system (e.g. *Proteus*,  
11 *Lactobacillus*, and *Enterococcus*) and their source (fly versus liver) could not be  
12 distinguished. These are likely very important bacteria to the system and may be  
13 symbionts of *Lucilia*. For instance, *Proteus*, which is attractive to *Lucilia*, is found in  
14 commensal relationship with *Lucilia*, and is not well eliminated by maggot debridement  
15 therapy (Fleischmann 2004; Nigam et al. 2006). This species is also known to produce  
16 “mirabilicides”, which kill some of the same bacteria *L. sericata* eliminates in maggot  
17 debridement therapy (Greenberg 1968; Mumcuoglu et al. 2001). For this reason, *Proteus*  
18 has been suggested as a potential means to enhance maggot debridement therapy.

19 Second, there was much more evidence for horizontal transmission of bacteria  
20 than there was for taxa that were trans-generationally inherited. Many bacterial genera  
21 (including *Staphylococcus*) are shared only by adult and aged liver samples, which  
22 suggest that these bacteria could have been deposited on the liver by the adult flies, and  
23 did not get completely consumed/eliminated by *L. sericata* larvae. This may be either  
24 because the maggots did not get enough feeding time to eliminate the bacteria or the  
25 maggots were not effective against these bacteria. This is important from a maggot  
26 debridement therapy point of view because if wounds are infected with these bacteria  
27 then most maggot treatment will not work on these wounds unless paired with other  
28 treatments like antibiotics. Such observations may support published literature on the  
29 effectiveness of maggot treatment of wound infections with the famous superbug  
30 Methicillin Resistant *Staphylococcus aureus* (MRSA), which are conflicting and  
31 inconclusive (Arora et al. 2011; Mumcuoglu 2001; van der Plas et al. 2008). One

1 possibility is that the larvae are capable of breaking down and disrupting biofilm  
2 formation by MRSA but prevent multiplication of planktonic bacteria and do not kill  
3 them (Cazander et al. 2013). Several genera are shared by adult and larval samples, and  
4 hence can be considered as potential trans-generationally inherited bacteria but it is not  
5 conclusive in this study because these genera are not exclusive to adult and larval  
6 samples. Further studies with labeled samples of this genus (as well as the ubiquitous  
7 genera) may provide further support for the inheritance patterns of these bacteria, as well  
8 as their spread into the environment by the flies.

9 Third, there appeared to be bacterial “winners” and “losers” in the experiment.  
10 There were several taxa that increased in abundance on the aged livers, even as they  
11 exhibited low abundances in the adult, larval and fresh liver samples (Fig. 7). These taxa  
12 included pathogens, suggesting that larval feeding on decomposition of liver as a  
13 resource may amplify the abundances of these microbes. For example, *Salmonella* was  
14 present at significantly low relative abundance (0.01%) in fresh liver sample but larval  
15 activities increased its relative abundance to significantly high level (2.01%) in aged liver  
16 sample. These observations suggest that these taxa are also not good candidates for  
17 removal by maggot debridement therapy, which is at odds with previously published  
18 reports that suggest that MDT is effective in controlling several drug resistant pathogens  
19 (e.g. *Salmonella*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Staphylococcus aureus*  
20 *etc.*) but often not Gram-negative bacteria (Cazander et al. 2013; Mumcuoglu 2001).  
21 However, there were also some taxa that were almost absent from larvae and aged livers  
22 (such as *Clostridium sensu stricto*). These are likely negatively impacted by the presence  
23 of larvae and their bacterial associates, as is observed with Gram-positives, and are better  
24 candidates for removal by bio-debridement than those that appear to be amplified in the  
25 presence of larvae (Fig. 6b and 7). These results suggest a need to match MDT to the  
26 situations that are most likely to result in successful wound debridement.

27 As a final experiment, given the importance of larval excretions to maggot  
28 therapy, the bacterial communities of the *L. sericata* salivary gland were evaluated. This  
29 yielded several interesting results. First, the microscopic assessment suggests that the  
30 salivary gland appears to be an inhospitable environment for bacteria, yielding few cells.  
31 Not surprisingly, the bacterial community of this organ appeared to differ from whole

1 carcass communities, most strikingly in the fact that *Proteus* appear in much higher  
2 abundances in the salivary gland. The taxa ubiquitously found in all life stages also  
3 appeared in the salivary gland, suggesting a possible role of this organ in the maintenance  
4 of some bacteria in the fly. In addition, there appears to be a balance between lactic acid  
5 producing Gram-positive and urease producing Gram-negative taxa in the salivary gland.  
6 It would be interesting to see if either or both routes of metabolism are important to the  
7 maintenance of these bacteria in the fly and if an imbalance between these metabolic  
8 groups yields negative consequences for the fly.

9         The overall goal of this research was to evaluate the bacterial communities  
10 associated with *Lucilia* species and to begin to characterize their inheritance patterns. The  
11 results of the study indicate that these flies harbor many of the bacterial taxa associated  
12 with the human gut and that most bacteria are maintained intra-generationally, with a  
13 considerable degree of turnover from generation to generation. There is little evidence in  
14 metagenomic analyses to support trans-generational inheritance of blow fly bacterial  
15 communities, though there is evidence that larvae appear to regulate their bacterial  
16 environment, resulting in bacterial “winners” and “losers” when maggots are present on a  
17 resource; some of which are pathogens. This study utilized 454-pyrosequencing  
18 approaches to highlight general trend in pathogen transmission by blow flies, but for  
19 more accurate individual pathogen transmission pattern, an qPCR based approach will be  
20 the best. Future studies should also focus on more detailed egg experiments from several  
21 generations of blow flies for elucidation of the mechanism behind vertical transmission of  
22 bacteria in blow flies.

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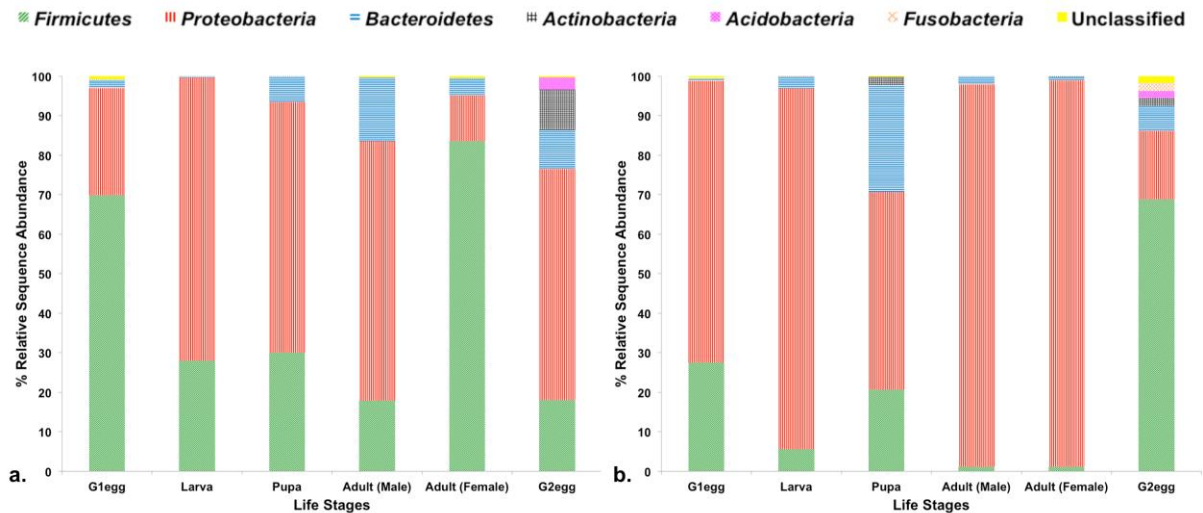
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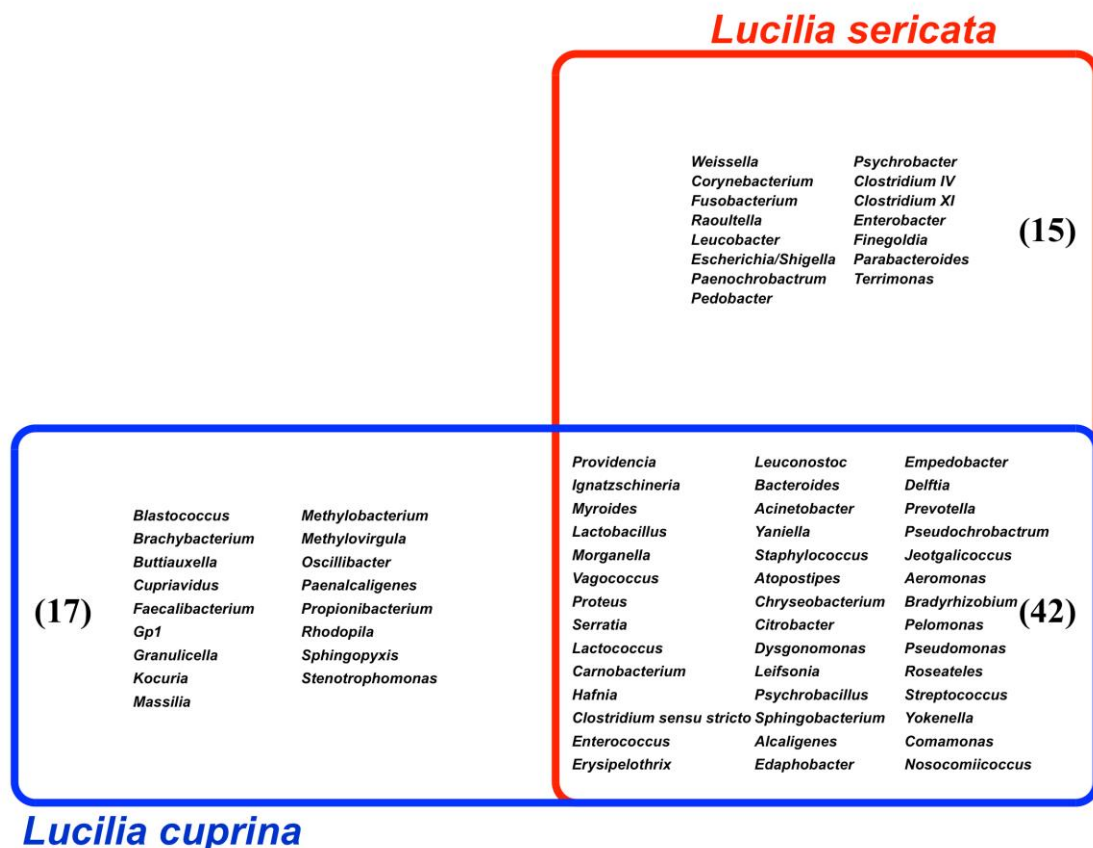
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**Table 1** Table showing bacterial diversity, evenness, and % coverage at three genetic distances.

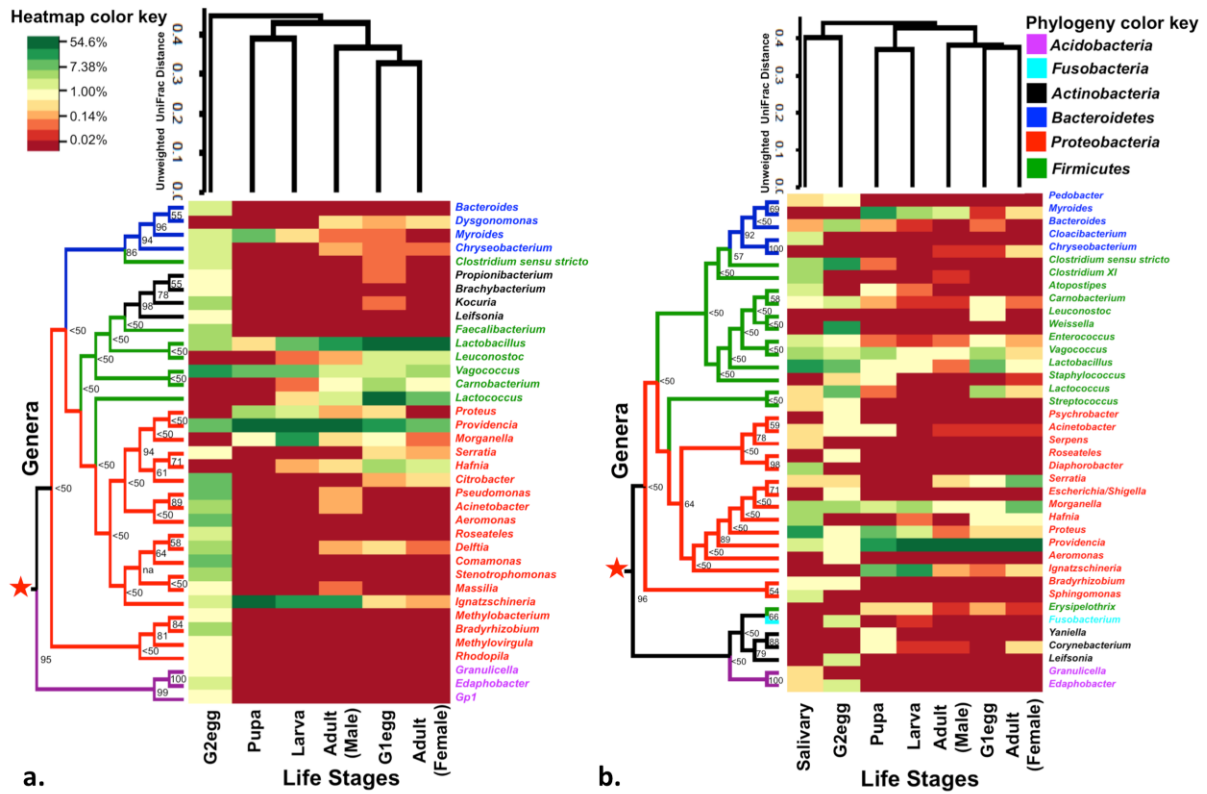
Species	Life stages	Shannon Index (H')			Shannon evenness (E)			Rarefaction (no. of OTUs)			Chao1 (no. of OTUs)			Coverage (%)		
		3%	5%	20%	3%	5%	20%	3%	5%	20%	3%	5%	20%	3%	5%	20%
<i>Lucilia cuprina</i>																
	G1egg	4.45	3.62	1.53	0.80	0.74	0.51	253	133	20	346	164	27	73	81	74
	Larva	3.64	3.05	1.16	0.70	0.66	0.45	187	104	13	269	124	13	70	84	98
	Pupa	4.08	3.20	1.55	0.74	0.66	0.52	241	131	20	371	197	27	65	66	74
	Adult (Male)	3.82	2.88	1.52	0.71	0.62	0.53	224	108	18	321	139	18	70	78	100
	Adult (Female)	4.45	3.43	1.16	0.75	0.66	0.47	378	174	12	526	215	12	72	81	100
	G2egg	4.13	3.85	2.69	0.93	0.92	0.93	84	66	18	134	116	18	62	57	100
<i>Lucilia sericata</i>																
	G1egg	3.65	2.86	1.06	0.66	0.60	0.38	243	119	16	390	152	16	62	78	100
	Larva	3.58	2.46	1.22	0.66	0.52	0.48	235	110	13	326	134	16	72	82	81
	Pupa	4.17	3.39	1.96	0.77	0.69	0.64	225	136	21	383	262	21	59	52	100
	Adult (Male)	2.45	1.18	0.26	0.52	0.30	0.11	111	48	11	158	67	12	70	71	92
	Adult (Female)	4.17	2.87	0.89	0.75	0.62	0.35	250	103	13	348	132	14	72	78	96
	G2egg	3.80	3.30	2.12	0.88	0.84	0.75	74	51	17	96	59	17	77	86	98



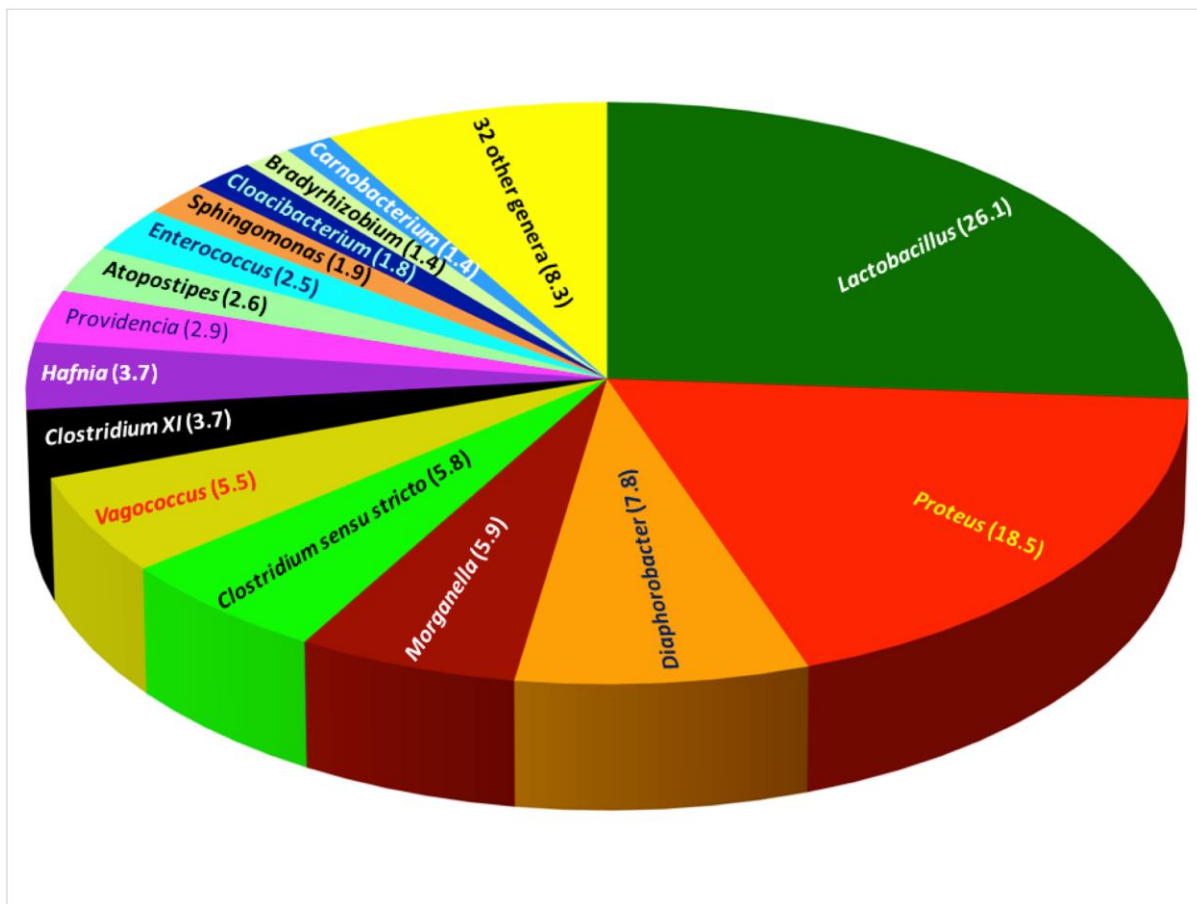
**Fig. 1** Phylum level bacterial sequence diversity from successive life stages of a.) *Lucilia cuprina*, and b.) *Lucilia sericata*. G1egg indicate first generation eggs and G2egg indicate second generation eggs.



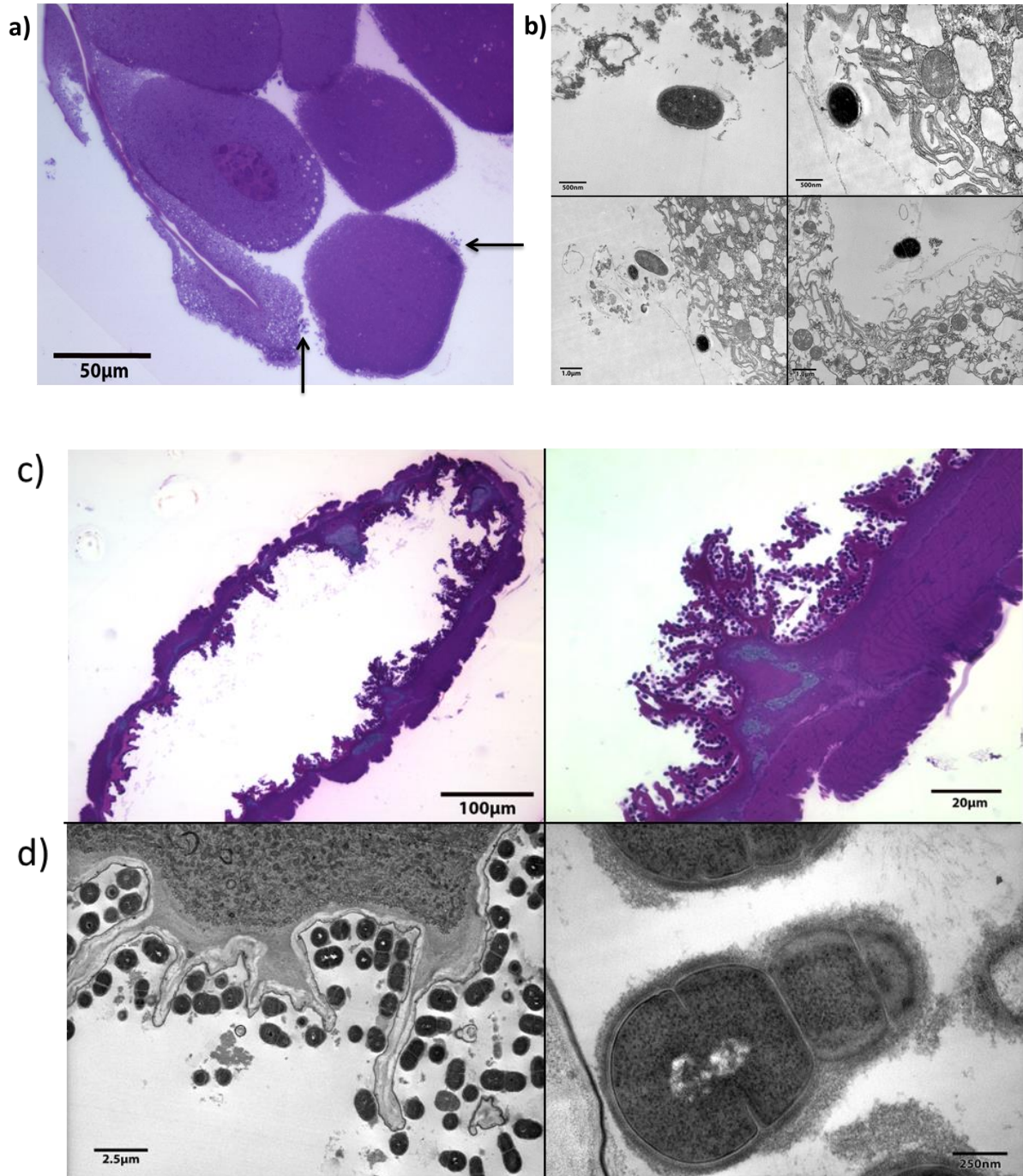
**Fig. 2** Venn diagram of bacterial genera associated with successive life stages of *Lucilia cuprina* (blue rectangle) and *Lucilia sericata* (red rectangle). Numbers in parentheses indicate total number of unique/shared bacteria associated with each species. Venn diagram was created using program Vennture (Martin et al. 2012).



**Fig. 3** Heatmap of dominant bacterial genera (% relative sequence abundance  $\geq 0.5$ ) associated with different life stages of a.) *Lucilia cuprina*, and b.) *Lucilia sericata*. Heatmap rows were clustered based on bootstrap neighbor-joining (NJ) tree of dominant genera associated with *L. cuprina* and *L. sericata*, and heatmap columns were clustered based on unweighted UniFrac distance of successive life stages of *L. cuprina* and *L. sericata*. For comparison purpose, % relative sequence abundance of salivary gland sample was also included along with successive life stages of *L. sericata*. AM= adult male; AF= adult female; G1egg= first generation eggs; G2egg= second generation eggs.

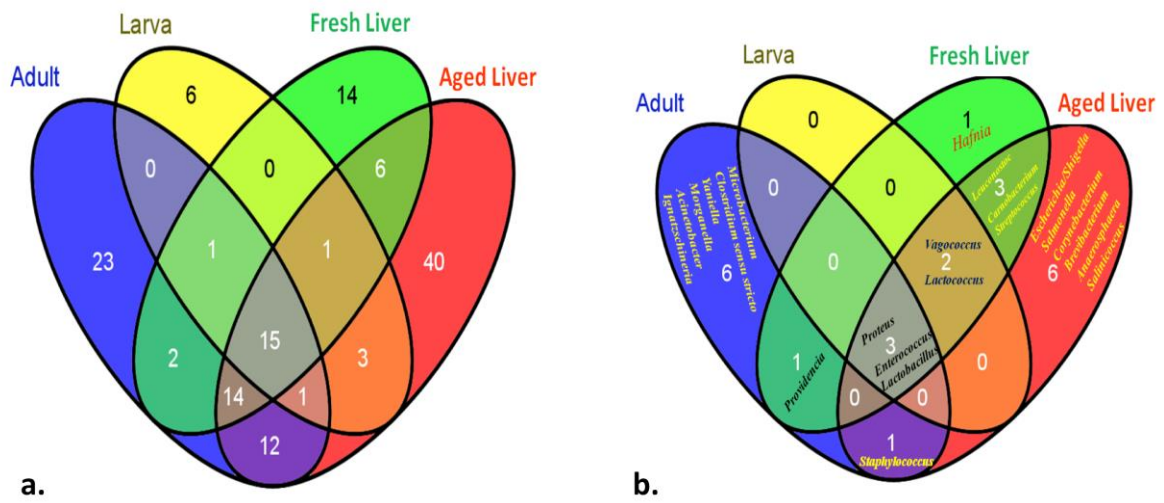


**Fig. 4** Pie diagram of classified bacterial genera associated with *Lucilia sericata* salivary gland. Numbers in parentheses indicate percent relative sequence abundance of each genus.

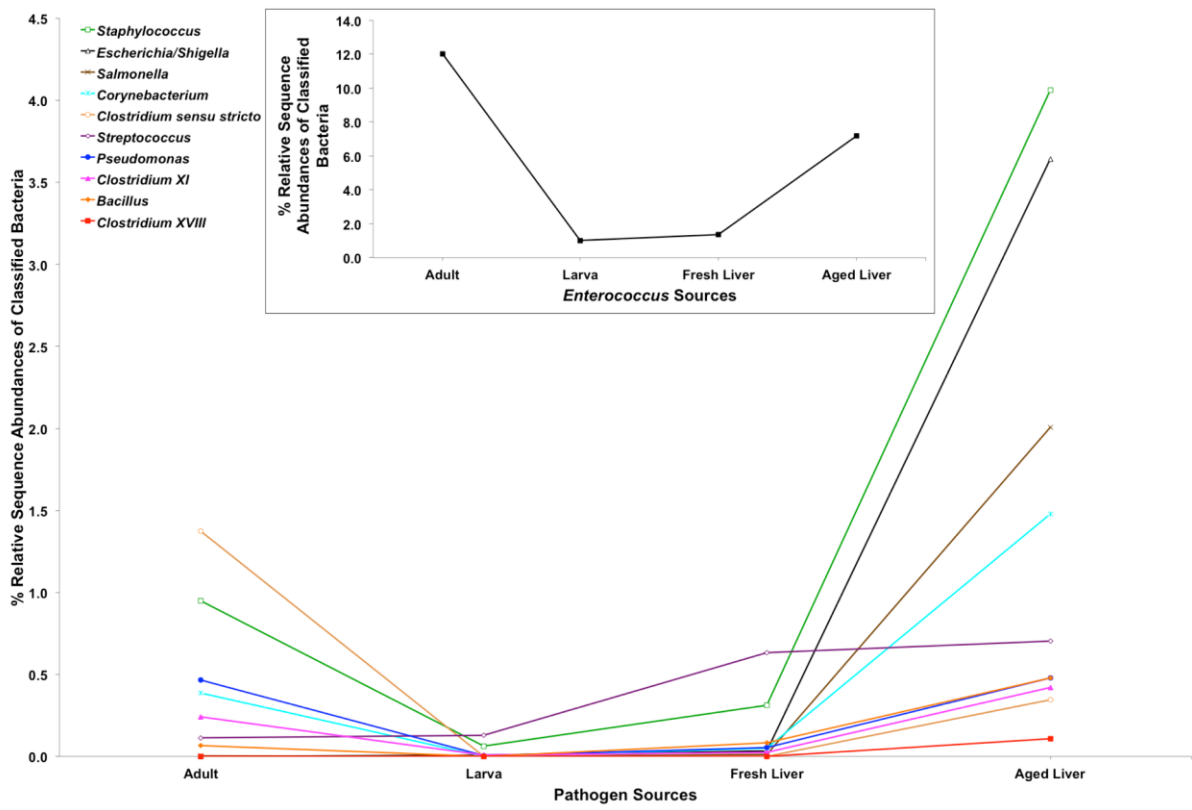


**Fig. 5** Salivary gland and crop images from third instar larvae of *Lucilia sericata* showing morphologies suggestive of Gram positive and Gram negative bacteria (arrows) a.) Light microscopy of 750 nm section of salivary gland (note that bacteria were found within the lumen of the gland, and not within the salivary cells themselves), b.) Transmission electron microscopy (TEM) of 70 nm section of salivary gland, c.) Light microscopy of 750 nm section of crop and d.) Transmission electron microscopy (TEM) of 70 nm section of crop. TEM sections were viewed in a Hitachi H7000 transmission electron microscope. Scale bars are shown.





**Fig. 6** Venn diagram of a.) all bacterial genera, and b.) bacterial genera that were present at 0.5% or higher relative abundance, associated with *Lucilia sericata* adult, *Lucilia sericata* larvae, fresh liver, and aged liver. Numbers indicate total number of unique and shared bacteria. Venn diagrams were created using web based program Venny (<http://bioinfogp.cnb.csic.es/tools/venny/>).



**Fig. 7** Line graph showing transmission of pathogenic bacteria. Graph in inset shows transmission of *Enterococcus*. Relative abundances of these bacteria were obtained from 454-sequences using RDP classifier.