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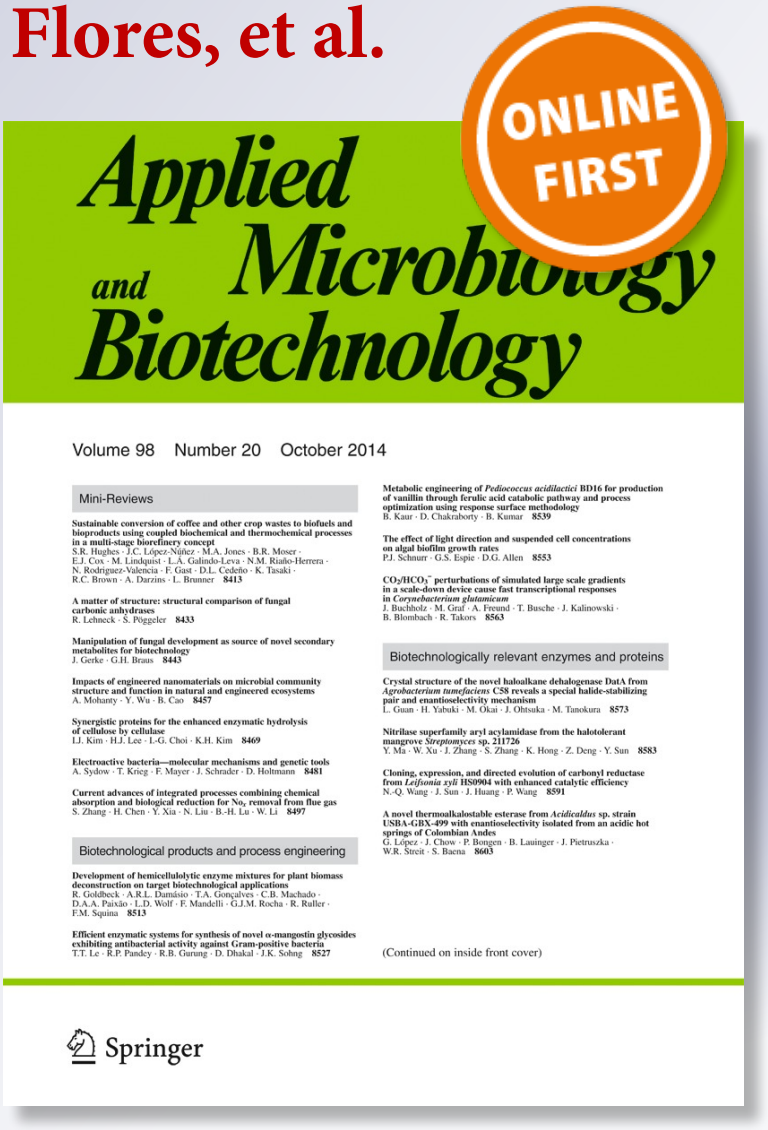
Applied Microbiology and Biotechnology

ISSN 0175-7598

Appl Microbiol Biotechnol
DOI 10.1007/s00253-014-6115-7



Springer

Appl Microbiol Biotechnol
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APPLIED MICROBIAL AND CELL PHYSIOLOGY

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

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Received: 28 July 2014 / Revised: 21 September 2014 / Accepted: 23 September 2014

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

Electronic supplementary material The online version of this article (doi:10.1007/s00253-014-6115-7) contains supplementary material, which is available to authorized users.

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Published online: 14 October 2014

information on bacterial communities associated with different life stages of *Lucilia* and their horizontal and trans-generational transmission, which may help in the development of better vector-borne disease management and MDT methods.

Keywords Microbial community · Blow flies · Maggot debridement therapy · Salivary gland · 454 sequencing

Introduction

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

Both species engage in myiasis, larval infestation of animal tissues (Ashworth and Wall 1994), which causes more than US$150 million of annual economic loss to the wool industry in Australia alone (Evans and Karlsson 2003). This behavior has beneficial uses though, as certain *L. sericata* strains (LB-01) are useful in maggot debridement therapy (MDT) (Mumcuoglu 2001; Sherman 2009). This practice uses sterilized larvae and their preference for dead tissue to debride non-healing necrotic wounds more efficiently than a surgeon or associated treatments (van der Plas et al. 2009). Given that the adults and larvae of the genus feed on feces and carrion (Clark et al. 2006) and live in constant association with decomposing matter, it is not surprising that their larval excretions and secretions (ES) have been demonstrated to possess antimicrobial properties (Cazander et al. 2009a; Harris et al. 2009; Kerridge et al. 2005; Mumcuoglu et al. 2001; Sherman et al. 2000). Larval ES has also recently been implicated with the ability to manipulate the development of microbial biofilms (Cazander et al. 2009b, 2010; Harris et al. 2009) and to stimulate wound angiogenesis (Bexfield et al. 2010), which may explain some of their antimicrobial and bio-surgical value. Accordingly, knowledge of microbial community associated with these flies can help ameliorate the negative perception of the approach (Steenvoorde et al. 2005) and promote their beneficial properties.

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

Identifying the potential microbial contaminants of experiments is important for deciphering the variation observed in research with these species. While maggot debridement therapy has been shown to decrease the prevalence of some microbes on a wound, other microbes are unaffected or increase in prevalence in association with treatment with *L. sericata* larvae (Jaklic et al. 2008). Sterile techniques for rearing *L. sericata* are well established (Mumcuoglu et al. 2001; Sherman and Tran 1994), but in some situations (e.g., use of non-sterile maggots instead of sterile maggots), MDT can also cause septicemia (Mumcuoglu 2001). In some situations, more than two species can colonize a wound premortem, which can complicate calculation of minimum postmortem interval estimation when using insect evidence in death investigations (Sanford et al. 2014). In all of these cases, knowledge of microbes associated with non-sterile larvae would aid in (1) identifying the likely sources of septicemia in the case of failed maggot debridement therapy, (2) interpreting the results of potentially non-sterile ES experiments, (3) identifying bacteria that are unaffected by the feeding of *Lucilia* larvae, and (4) identifying bacteria that attract different blow flies for oviposition pre- or postmortem.

These considerations raise several questions regarding potential bacterial communities associated with these important blow flies: (1) What bacteria are associated with these species, and how similar are the bacterial communities associated with each species?; (2) What bacteria are likely to be trans-generationally transmitted and what bacteria are likely to be horizontally transmitted?; and (3) What bacteria are amplified

or eliminated by larval feeding? To address these questions, we conducted a survey of bacterial communities associated with these sister species using 16S rDNA 454 pyrosequencing.

Materials and methods

Fly colony maintenance

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

Sample collection

Fly life stages

Generationally related eggs, larvae, pupae, and adults (male and female) were raised in the same environment on raw beef liver. Each of the experiments was done with one replicate per species, as the goal was to (1) categorize bacteria associated with the flies and (2) determine if it appeared likely that bacteria were mostly horizontally or trans-generationally inherited. Approximately 0.5 g eggs (first-generation eggs or G1egg) were removed for DNA extraction. The remainder of the eggs was left to hatch and was harvested sequentially as the flies developed. The resulting third instar larvae (larva), pupae (pupa), adult males (AM), adult females (AF), and second-generation eggs (G2egg) were randomly collected and frozen at −80 °C until DNA extraction could be performed.

Salivary gland removal protocol

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

Transmission electron microscopy

Salivary glands were preserved in a fixative consisting of 3 % glutaraldehyde, 2 % paraformaldehyde, and 12 % picric acid prepared in 50 mM phosphate buffer, pH 7.4, and 50 mM sucrose. Salivary glands in fixative were incubated at room temperature for 60 min then held at 4 °C. Subsequent to primary fixation, salivary glands were postfixed for 2 h at 4 °C in 1 % osmium tetroxide prepared in 100 mM phosphate buffer, pH 7.4, 100 mM sucrose, and 50 mM $K_4Fe(CN)_6$ (potassium ferricyanide). After osmication, samples were rinsed at 4 °C in 50 mM phosphate buffer, pH 7.4, containing 50 mM sucrose followed by eight rinses in 4 °C distilled H_2O over the course of 2 h, then post-staining overnight at 4 °C in 0.5 % uranyl acetate. Following post-staining, samples were rinsed in 4 °C distilled H_2O and dehydrated in a graded ethanol series and acetone. Dehydration was followed by infiltration and embedding in Mollenhauer's formulation of epoxy resin (Mollenhauer 1964). Thin TEM sections, 70 nm, were cut and stained using 1 % uranyl acetate and lead citrate then viewed in a Hitachi H7000 Transmission Electron Microscope. Sections, 750 nm, for light microscopy were stained with either 0.05 % toluidine blue or a mixture of basic fuchsin and toluidine blue (Multiple Stain, Polysciences, Warrington, PA, USA).

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

To better understand the dynamics of bacterial exchange between the environment and *L. sericata*, an experiment was conducted to allow adult flies to oviposit on three different commercial sources of liver (previously frozen at −20 °C) and follow the flies that developed (Fig. S1). The bacteria from the adults and liver prior to oviposition and from third instars and the liver after development were evaluated. The three liver sources were collected from different supply chains (*x*, *y*, and *z*) to maximize the variation in liver-associated microbes. Four 0.25 g replicate samples were randomly collected from each liver sample prior to exposure to adult flies (fresh liver) and after use by and removal of flies (aged liver). Four replicate samples each of six (three male and three female) adult flies prior to access to the liver (adult) and of 0.25 g third instar larvae that were oviposited and had grown on the specific liver sources (larvae) were randomly collected. Samples were

stored at −80 °C until DNA extraction was performed. The experiment was replicated three times.

DNA extraction

DNA extractions were performed from 0.25 g liver tissue, 0.25 g eggs (1 h old), two larvae (7-day old), two pupae, and two newly emerged adults. These samples were selected randomly and whole insect specimens were homogenized in 1.5 mL PBS. Briefly, homogenized samples were placed in 1.5 mL microcentrifuge tubes with 500 μL Tris-EDTA (pH= 8), 50 μL 10 % SDS, 3 μL proteinase K (20 mg/mL), and 1.5 μL of lysozyme (50 mg/mL) and then incubated with shaking (900 rpm) at 56 °C in a water bath. After 1 h of incubation, 100 μL NaCl (5 M) and 80 μL CTAB extraction solution (Teknova, USA) were added and samples thoroughly mixed and incubated at 65 °C for 10 min. Sequential extraction in a 1× volume was performed using phenol (pH 8.0), phenol/chloroform/isoamyl alcohol (25:24:1), and chloroform/isoamyl alcohol (24:1) by centrifugation at 6000×*g* for 6 min. The DNA was precipitated in 0.7 volume of isopropanol, washed twice in 70 % ethanol, dissolved in nuclease-free water, and quantified by spectrophotometry. Extracted DNA was aliquoted and sent to the Research and Testing Laboratory (http://www.researchandtesting.com/) for 16S rDNA 454 pyrosequencing using universal bacterial primer pair 27F (5′-GAGTTTGATCNTGGCTCAG) and 519R (5′-GTNTTACNGCGGCKGCTG) by bacterial tag-encoded FLX-Titanium pyrosequencing (bTEFAP) method (Dowd et al. 2008) in Genome Sequencer FLX System (Roche, Nutley, NJ, USA). All FLX-related procedures were performed following Genome Sequencer FLX System manufacturer's instructions (Roche, Nutley, NJ, USA).

Pyrosequencing data analysis

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

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

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

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

Diversity indices were calculated using tools available in RDP pyrosequencing pipeline (http://pyro.cme.msu.edu/). Rarefaction curves were generated in Excel 2007 (Microsoft Corporation, Redmond, WA) using results obtained from the tools Aligner, complete linkage clustering, and rarefaction of RDP pyrosequencing pipeline (Cole et al. 2009) (http://pyro.

cme.msu.edu/; accessed on October 23, 2012). Shannon (1948) and Chao 1 (Chao and Bunge 2002) indices were calculated using the tool Shannon and Chao1 index of RDP pyrosequencing pipeline (Cole et al. 2009) (http://pyro.cme.msu.edu/; accessed on October 23, 2012). Percentage coverage of species richness was calculated from rarefaction and Chao1 indices using the method as described in Zheng et al. (2013). All raw sequence files were submitted to the Sequence Read Archive (SRA). Study accession no. PRJEB6623 can be used for the retrieval of raw sequences used in this study.

Results

General characteristics of 454 sequences

This study produced 29,792 chimera-free bacterial sequences with an average length of 296 bp. These samples came from successive life stages of the blow fly sister species *L. cuprina* and *L. sericata*. The number of sequences obtained from G1egg, larvae, pupae, adult (male), adult (female), and G2egg samples were 1965, 1961, 3081, 2415, 4451, and 234 in *L. cuprina* and 3053, 4113, 1752, 2583, 3896, and 288 in *L. sericata*, respectively. In *L. cuprina,* approximately 99.7, 98.8, 98.1, 92.7, and 82 % of all sequences were classified with ≥80.0 % bootstrap support into 5 phyla, 11 classes, 17 orders, 42 families, and 59 genera, respectively. On the other hand, in *L. sericata*, approximately 99.9, 99.7, 99.4, 98.2, and 76.5 % of all sequences were classified with ≥80.0 % bootstrap support into 7 phyla, 13 classes, 22 orders, 49 families, and 83 genera, respectively. Additionally, 1283, 13,347, 22,790, 17,261, and 12,695 sequences were also obtained from *L. sericata* salivary gland, *L. sericata* adults, *L. sericata* third instar larvae, fresh liver, and aged liver, respectively (see Fig. S1 for experimental design). In these samples, approximately 99.6, 99.5, 97.9, 94.8, and 77.0 % of all sequences were classified with ≥80.0 % bootstrap support into 6 phyla, 11 classes, 20 orders, 38 families, and 47 genera, respectively.

Taxonomic distribution of 454 sequences

The majority of sequences (>99 %) collected from successive life stages of *Lucilia* belonged to the phyla *Proteobacteria*, *Firmicutes*, and *Bacteroidetes* (Fig. 1; Table S1). Phylum level relative sequence abundances associated with male and female adult *L. sericata* flies were almost the same (mainly *Proteobacteria*), but this was not true with *L. cuprina* male and female adults (Fig. 1; Table S1). *Acidobacteria* and *Actinobacteria* were mainly associated with G2egg in both species. *Fusobacteria* were mainly present in *L. sericata* G2egg samples. Similarly, more than 90 % of all classified sequences across all life stages belong to the classes *Gammaproteobacteria*, *Bacilli*, and *Flavobacteria* and orders *Enterobacteriales*, *Xanthomonadales*, and *Lactobacillales* in both *Lucilia* species (Table S1). Additionally, *Flavobacteriales* and *Bacillales* were present at relatively higher sequence abundances in pupal samples of both *Lucilia* species. At the family level, *Enterobacteriaceae*, *Xanthomonadaceae*, *Lactobacillaceae*, and *Enterococcaceae* were present in high numbers across all life stages of *Lucilia* spp. (Table S1). *Flavobacteriaceae* were mainly present in the pupal stage of both *Lucilia* species (Table S1). Although the

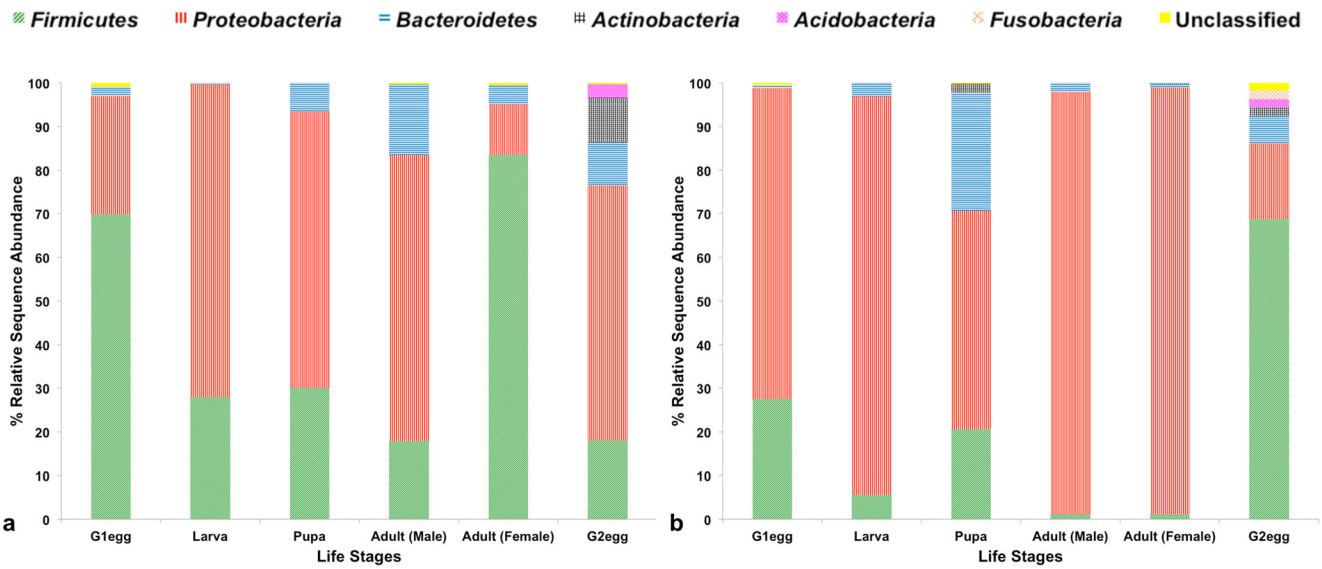

Fig. 1 Phylum-level bacterial sequence diversity from successive life stages of **a** *Lucilia cuprina* and **b** *Lucilia sericata. G1egg* indicates first-generation eggs and *G2egg* indicates second-generation eggs

blow fly sister species shared the majority of their classified genera (42 genera), there were some that were only observed in one species (Fig. 2). Of the bacterial genera identified, *Lactobacillus* (25 %), *Providencia* (24 %), *Ignatzschineria* (10 %), *Lactococcus* (8 %), and *Vagococcus* (4.4 %) were the five most dominant genera associated with *L. cuprina*, whereas *Providencia* (53 %), *Ignatzschineria* (5 %), *Myroides* (4 %), *Lactobacillus* (3 %), and *Morganella* (2.6 %) were the five most dominant genera associated with *L. sericata* (Fig. 3; Table S1). Pupae of both blow fly species had relatively high abundances of *Myroides*.

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

Bacterial richness and diversity indices

In *L. cuprina*, bacterial diversity at species (3 % sequence divergence) and genus (5 % sequence divergence) levels was similar in all life stages, but at the phylum level (20 % genetic divergence), diversity was relatively higher in G2egg than any other life stages (Table 1). In *L. sericata*, at species (3 % sequence divergence) and genus (5 % sequence divergence) levels, bacterial diversity was almost the same in all life stages, except in male adult samples, where bacterial diversity was lowest compared to all other life stage samples at all sequence divergences. At 20 % sequence divergence, bacterial diversity was relatively higher in G2egg and pupal samples. Similar trends were observed with

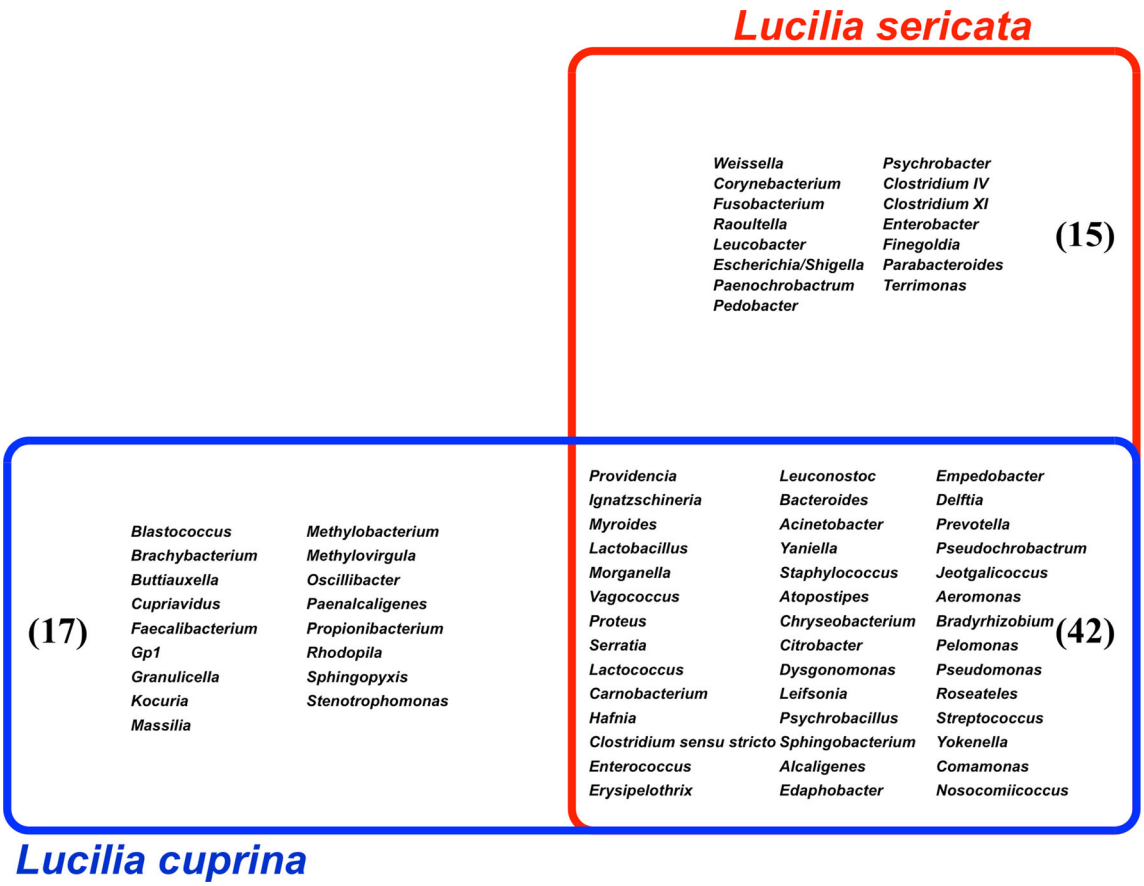

Fig. 2 Venn diagram of bacterial genera associated with successive life stages of *Lucilia cuprina* (*blue rectangle*) and *Lucilia sericata* (*red rectangle*). *Numbers in parentheses* indicate the total number of unique/shared bacteria associated with each species. Venn diagram was created using program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

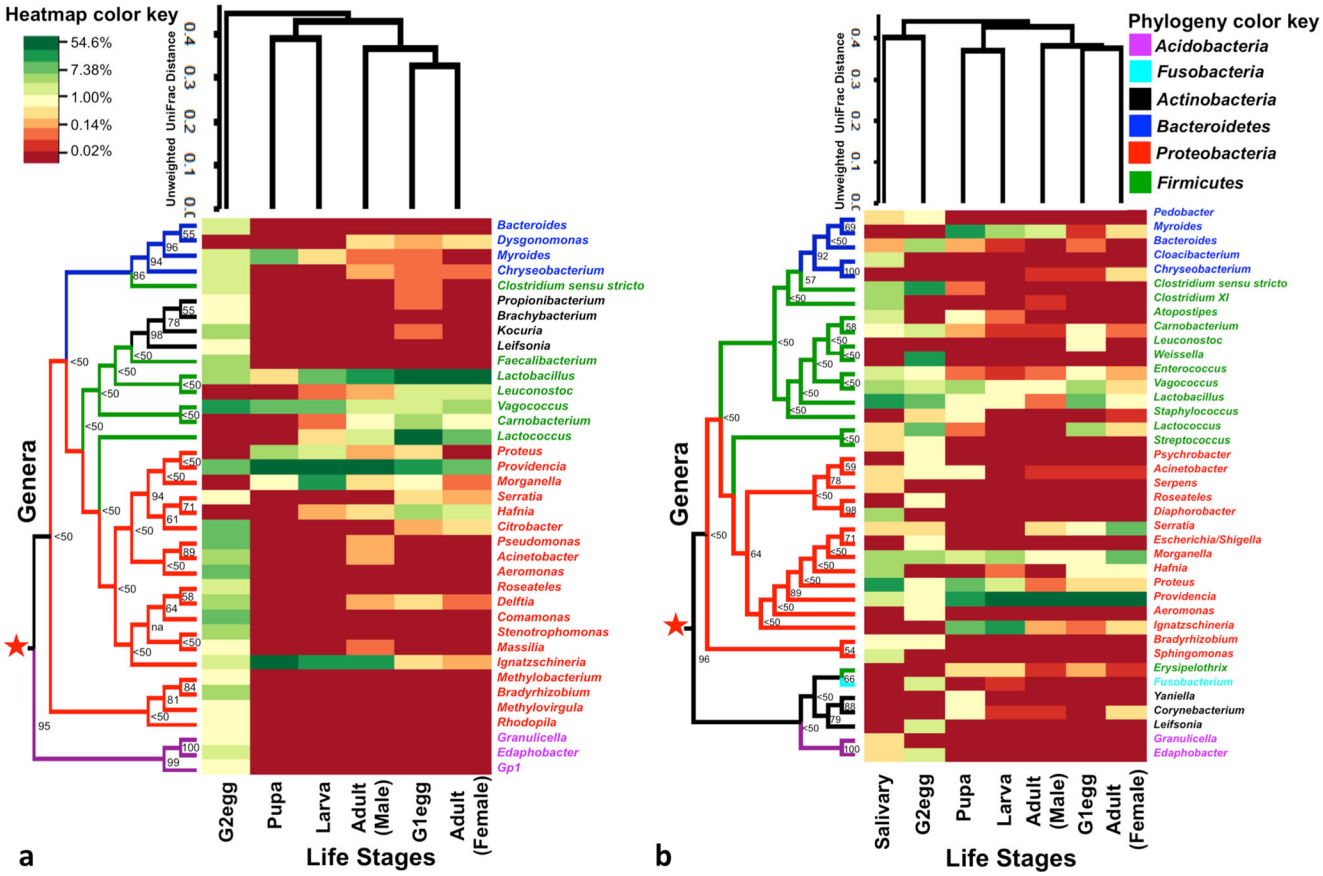

Fig. 3 Heatmap of dominant bacterial genera (% relative sequence abundance ≥0.5) associated with different life stages of **a** *Lucilia cuprina* and **b** *Lucilia sericata*. Heatmap rows were clustered based on bootstrap neighbor-joining (NJ) tree of dominant genera associated with *L. cuprina* and *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*. *G1egg* first-generation eggs, *G2egg* second-generation eggs

rarefaction and Chao1 estimators (Table 1, Fig. S3). Sequencing effort covered more than 60 % of bacterial diversity at species level (except *L. sericata* pupa), more than 66 % at genus level (except G2egg in *L. cuprina* and pupal samples in *L. sericata*), and more than 80 % at phylum level (except G1egg and pupa in *L. cuprina*).

Table 1 A 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 %
Lucilia cuprina	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
Lucilia sericata	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

Bacteria in the salivary glands of *L. sericata*

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

Trans-generationally and horizontally transmitted bacteria

Bacterial communities associated with fresh liver and aged liver samples were more similar to each other than to either *L. sericata* adults that landed, ate, and oviposited on the liver or the *L. sericata* larvae that had fed upon the liver in both weighted and unweighted FastUniFrac-based clustering (Fig. S4). Adult and aged liver samples shared 12 bacterial genera that were not present in larval and fresh liver samples. On the other hand, *L. sericata* adult and larval samples did not share any bacteria that were not present in other samples. A total of 15 genera were shared by all samples (adult, larva, fresh liver, and aged liver). Out of the 15 genera, *Proteus*, *Enterococcus*, and *Lactobacillus* were the dominant genera that were present in all samples (Fig. 6a, b). Several pathogenic genera were also present in adult and/or fresh liver samples, which either got amplified or reduced by larval activities (Fig. 7).

Discussion

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

The first part of the study evaluated an un-replicated (at the level of fly species) developmental time series of fly-associated bacterial communities, starting with eggs, proceeding throughout development, and culminating in a second generation of eggs. These data are useful for establishing the presence of certain members of the bacterial communities, but absence and concentration information should be carefully

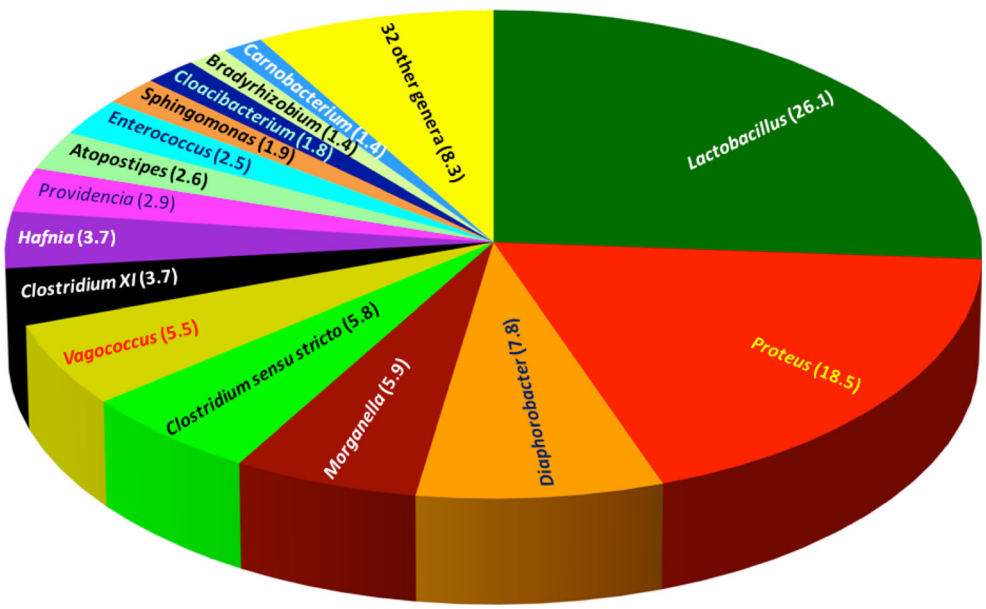

Fig. 4 Pie diagram of classified bacterial genera associated with *Lucilia sericata* salivary gland. Numbers in parentheses indicate the percent relative sequence abundance of each genus

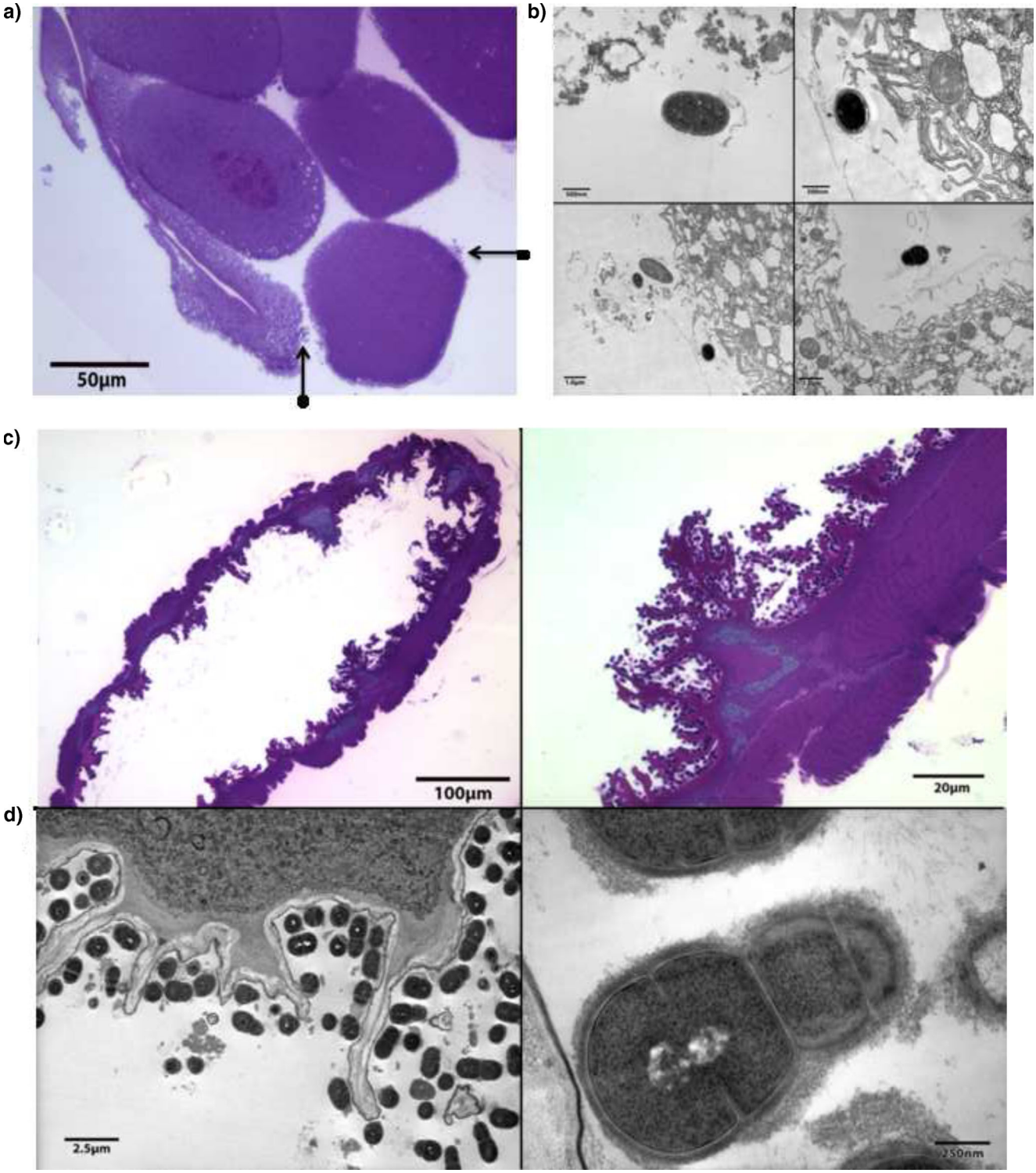

Fig. 5 Salivary gland and crop images from third instar larvae of *Lucilia sericata* showing morphologies suggestive of Gram-positive and Gram-negative bacteria (*arrows*). **a** Light microscopy of a 750-nm section of the salivary gland (note that bacteria were found within the lumen of the gland and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

considered with the fact that replication was not done per time point per species. With this caveat in mind, it is interesting to note that many of the same bacteria appeared in both time series, it was clear that there was a different community composition associated with species, representing numerous taxa, mostly from those phyla found in the human (Backhed

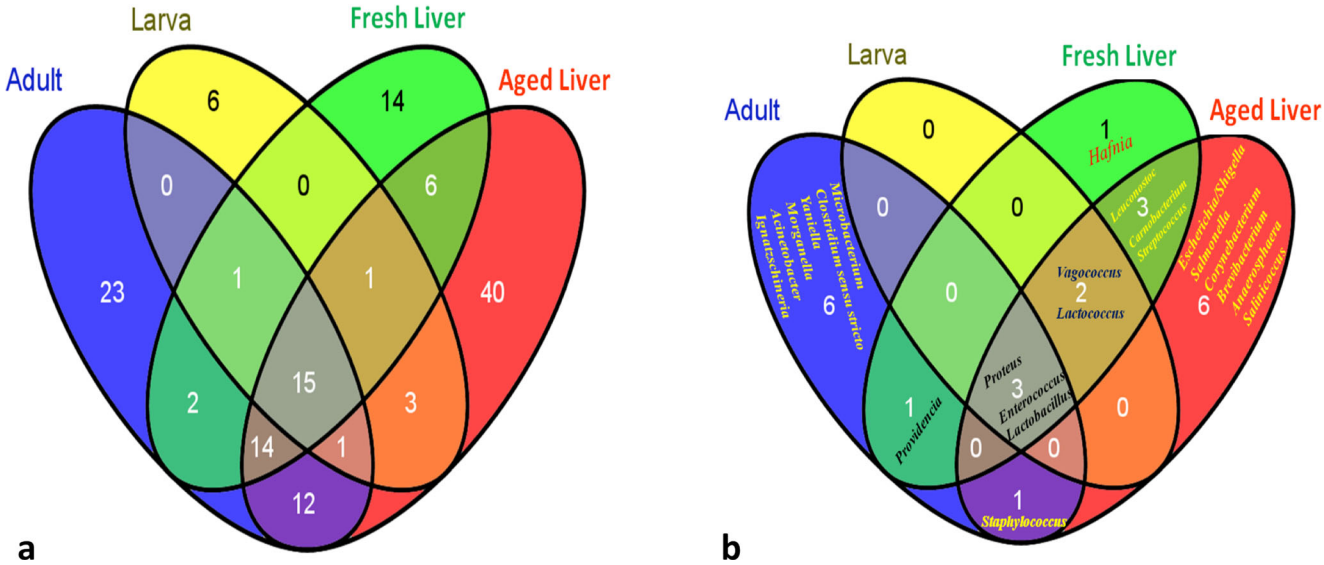

Fig. 6 Venn diagram of **a** all bacterial genera and **b** bacterial genera that were present at 0.5 % or higher relative abundance, associated with *Lucilia sericata* adult, *Lucilia sericata* larvae, fresh liver, and aged liver. The *numbers* indicate the total number of unique and shared bacteria.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

et al. 2005) and insect (Gupta et al. 2012, 2014; Wei et al. 2014b; Zheng et al. 2013) gut. Relative abundances appeared to differ between species, but this portion of the study was not replicated within species, making it impossible to differentiate replicate effects from species effects. Given that limitation, both time series observations still demonstrated that each

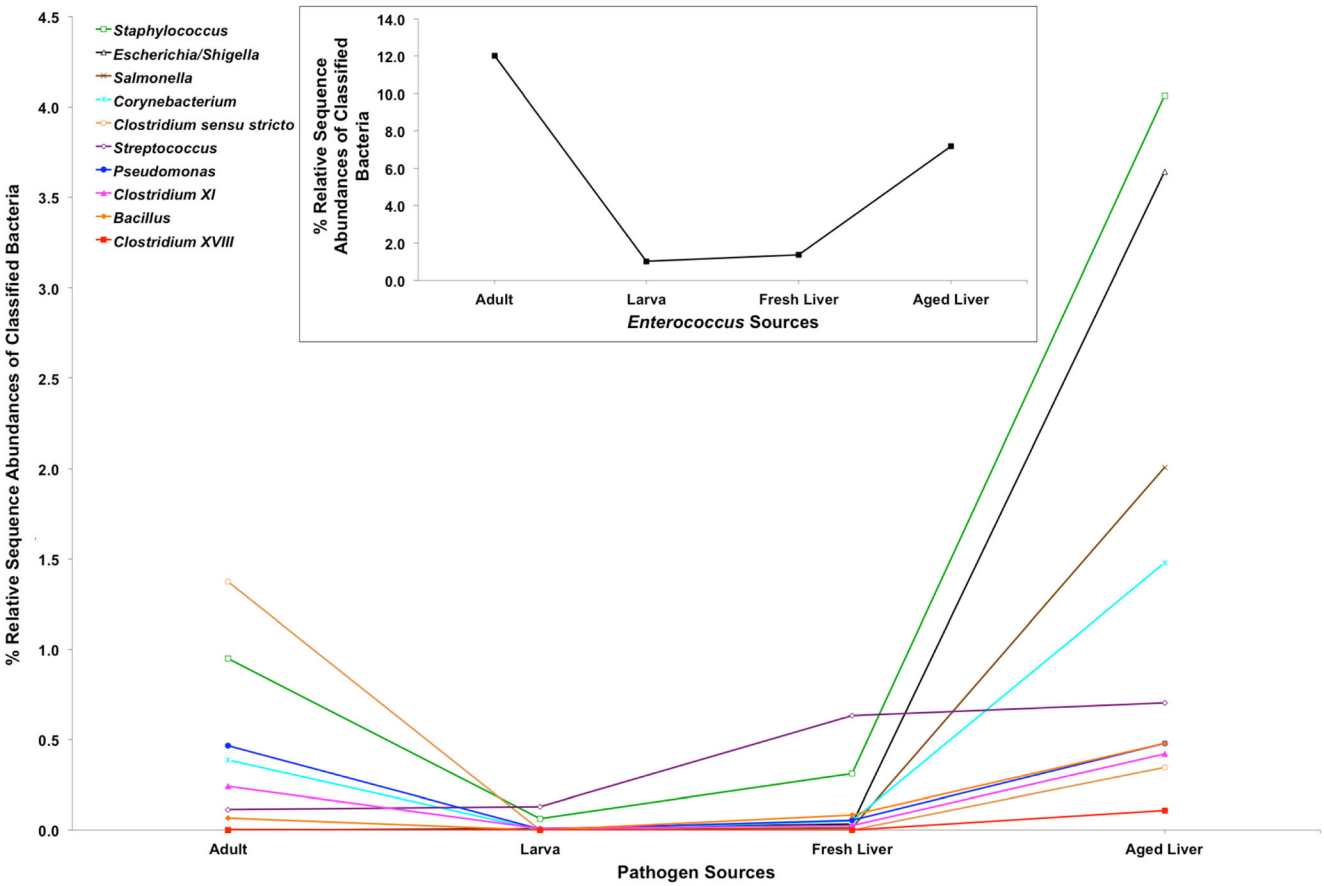

Fig. 7 A line graph showing the transmission of pathogenic bacteria. The graph in the inset shows transmission of *Enterococcus*. Relative abundances of these bacteria were obtained from 454 sequences using RDP classifier

sister species of *Lucilia* consists of some putatively unique and many shared bacterial genera, with a large turnover in community occurring for both species at oviposition.

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

At the commencement of a new generation, bacterial communities associated with eggs were considerably altered from the previous generation, even from that of the maternal bacterial communities. Trans-generationally inherited bacteria in G2egg might have come either from the mother or from the environment. In both *Lucilia* species, G2egg samples differed from other life stages mainly because of relatively high abundance of *Acidobacteria* and *Actinobacteria* (Fig. 1). The genome of *Acidobacteria* contains several cellulose and protein-synthesizing genes (Ward et al. 2009). A network of bacterial celluloses can produce biofilm, retain water under dry conditions, and help in aeration. All these functions of the network of celluloses most probably contribute in egg structure and protection of eggs from desiccation (Ward et al. 2009). Members of *Actinobacteria* are known to produce several antimicrobial bioactive compounds, which may be protecting egg from harmful bacteria and fungi (Mahajan and Balachandran 2012; Raghava Rao et al. 2012). This may also be a reason why we see relatively less bacterial sequences in egg samples compared to other life stage samples. This was seen previously in the black soldier fly, *Hermetia illucens* (L.) (*Diptera: Stratiomyidae*) (Zheng et al. 2013), and it remains to be seen if this is a property of the experimental design or a feature of carrion fly biology. *Fusobacteria*, which is a causative agent for bacteremia, was observed only in the G2egg of *L. sericata*, which suggests to us that these bacteria may be the responsible agent for the fatal myiasis, sometimes caused by *L. sericata* (Henry et al. 1983; Mowlavi et al. 2011).

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

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

First, in unweighted FastUniFrac clustering, the bacterial community structures associated with *L. sericata* adults were more similar to fresh and aged liver samples than to larval sample, whereas in weighted FastUniFrac clustering, bacterial community structures associated with fresh and aged liver samples were more similar to larval samples than to adult samples (Fig. S4). Because weighted FastUniFrac clustering is based both on bacterial composition and quantity (compared to just bacterial composition in unweighted FastUniFrac clustering), a close relationship between liver and larval samples in weighted FastUniFrac clustering is most probably because of similar numbers and types of taxa in these samples, suggesting convergence in communities due either to larval manipulation of the bacterial community on the liver or the ability of larvae to persist in the community found on the liver without needing to regulate its own community. For example, *Vagococcus* and *Lactobacillus* were present at very high % relative abundances (>25 %) in larval and liver samples, but their relative abundances were significantly low (<1 %) in adult samples.

Many bacterial genera are common throughout the system (e.g., *Proteus*, *Lactobacillus*, and *Enterococcus*) and their source (fly versus liver) could not be distinguished. These are likely very important bacteria to the system and may be

symbionts of *Lucilia*. For instance, *Proteus,* which is attractive to *Lucilia*, is found in commensal relationship with *Lucilia* and is not well eliminated by maggot debridement therapy (Fleischmann 2004; Nigam et al. 2006). This species is also known to produce "mirabilicides," which kill some of the same bacteria *L. sericata* eliminates in maggot debridement therapy (Greenberg 1968; Mumcuoglu et al. 2001). For this reason, *Proteus* has been suggested as a potential means to enhance maggot debridement therapy.

Second, there was much more evidence for horizontal transmission of bacteria than there was for taxa that were trans-generationally inherited. Many bacterial genera (including *Staphylococcus*) are shared only by adult and aged liver samples, which suggest that these bacteria could have been deposited on the liver by the adult flies, and did not get completely consumed/eliminated by *L. sericata* larvae. This may be either because the maggots did not get enough feeding time to eliminate the bacteria or the maggots were not effective against these bacteria. This is important from a maggot debridement therapy point of view because if wounds are infected with these bacteria, then most maggot treatment will not work on these wounds unless paired with other treatments like antibiotics. Such observations may support published literature on the effectiveness of maggot treatment of wound infections with the famous superbug methicillin-resistant *Staphylococcus aureus* (MRSA), which are conflicting and inconclusive (Arora et al. 2011; Mumcuoglu 2001; van der Plas et al. 2008). One possibility is that the larvae are capable of breaking down and disrupting biofilm formation by MRSA but prevent multiplication of planktonic bacteria and do not kill them (Cazander et al. 2013). Several genera are shared by adult and larval samples and hence can be considered as potential trans-generationally inherited bacteria but it is not conclusive in this study because these genera are not exclusive to adult and larval samples. Further studies with labeled samples of this genus (as well as the ubiquitous genera) may provide further support for the inheritance patterns of these bacteria, as well as their spread into the environment by the flies.

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

As a final experiment, given the importance of larval excretions to maggot therapy, the bacterial communities of the *L. sericata* salivary gland were evaluated. This yielded several interesting results. First, the microscopic assessment suggests that the salivary gland appears to be an inhospitable environment for bacteria, yielding few cells. Not surprisingly, the bacterial community of this organ appeared to differ from whole carcass communities, most strikingly in the fact that *Proteus* appear in much higher abundances in the salivary gland. The taxa ubiquitously found in all life stages also appeared in the salivary gland, suggesting a possible role of this organ in the maintenance of some bacteria in the fly. In addition, there appears to be a balance between lactic acid-producing Gram-positive and urease-producing Gram-negative taxa in the salivary gland. It would be interesting to see if either or both routes of metabolism are important to the maintenance of these bacteria in the fly and if an imbalance between these metabolic groups yields negative consequences for the fly.

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

Acknowledgments The authors would like to thank Dr. Robert Droleskey for his assistance with transmission electron microscopy of the salivary gland. Funding for B.S., A.M.T., L.Z., A.T.F., M.F., and J.K.T. was provided partially by the Texas Agrilife Research and the College of Agriculture and Life Sciences at Texas A&M University, College Station, TX. Additional funding for B.S., A.M.T., T.L.C., and J.K.T. was provided by the National Institute of Justice, Office of Justice Programs, U.S. Department of Justice through Grant 2010-DN-BX-K243. B.S. was also supported by a start up fund from the College of Humanities and Sciences of Virginia Commonwealth University, Richmond, VA. We also thank anonymous reviewers for their suggestions that improved this article substantially. Points of view in this document are those of the authors and do not necessarily represent the official position or policies of the U.S. Department of Justice, Department of the Army, Department of Defense, or U.S. Government. Mention of trade names, companies, or commercial products in this publication is solely for the purpose of providing specific information and does not imply recommendation or endorsement of the products by the U.S. Department of Agriculture.

Conflict of interest The authors have no conflict of interest.

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