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Microbial Communities Associated With Stable Fly (Diptera: Muscidae) Larvae and Their Developmental Substrates

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Abstract

Bacteria are essential for stable fly (*Stomoxys calcitrans* (L.)) larval survival and development, but little is known about the innate microbial communities of stable flies, and it is not known if their varied dietary substrates influence their gut microbial communities. This investigation utilized 454 sequencing of 16S and 18S amplicons to characterize and compare the bacterial and eukaryotic microbial communities in stable fly larvae and their developmental substrates. The microbial community of the third-instar stable fly larvae is unambiguously distinct from the microbial community of the supporting substrate, with bacterial communities from larvae reared on different substrates more similar to each other than to the communities from their individual supporting substrates. Bacterial genera that were more abundant proportionally in larvae compared to their substrates were *Erysipelothrix*, *Dysgonomonas*, *Ignatzschineria* (Gammaproteobacteria), and *Campylobacter* (Epsilonprotobacteria). The alphaproteobacteria *Devosia*, *Brevundimonas*, *Sphingopyxix*, and *Paracoccus* were more abundant proportionally in field substrates compared to their larvae. The main genera responsible for differences between the positive and negative field substrates were *Dysgonomonas* and *Proteiniphilum*. In contrast to *Dysgonomonas*, *Proteiniphilum* was more abundant in substrate than in the larvae. A large number of sequences were assigned to an unclassified protest of the superphylum Alveolata in larvae and their substrate. Microscopy validated these findings and a previously undescribed gregarine (phylum Apicomplexa, class Conoidasida) was identified in stable fly larvae and adults.

Key words: *Stomoxys calcitrans*, bacteria, fungi, gregarine, 454 sequencing

Adult stable flies are serious hematophagous pests of pasture and feedlot cattle. Their painful bites distress the animals, prompting host defensive behaviors such as bunching and wading in water, causing concerns related to animal welfare, water quality, and reduced weight gains (Campbell et al. 1987, Taylor et al. 2012).

The most effective method of stable fly control is identifying and removing substrates that support development. Providing guidance to farmers on eliminating stable fly larval habitat is difficult, in part, because the environmental conditions that foster stable fly growth and development have not been established. Stable flies develop in a diverse array of substrates that generally include moist, decaying vegetation such as animal bedding, silage, spilled hay, lawn clippings, and various crop residues (i.e., peanut, pineapple, corn, and sugar cane; Ware 1966, Williams et al. 1980, Talley et al. 2009, Schmidtmann 1991, Solorzano et al. 2015). Physiochemical measurements have provided some insight into why stable flies are able to develop in such diverse substrates, but it is evident that microbes colonizing the substrate also have impacts (Giles et al. 2008, Wienhold and Taylor 2012, Friesen et al. 2016b). Bacteria are essential for stable fly larval survival and development (Lysyk et al. 1999, Romero et al. 2006, Albuquerque and Zurek 2014). However, little is known about the innate microbial communities of these flies, and it is not known to what extent dietary substrate influences the gut microbial communities of these insects.

To explore these questions, this study was designed to examine the microbial communities of larvae and their supporting substrates. The objectives of this investigation were to 1.) Characterize the microbial communities of calf bedding, a common source of stable fly development, as well as artificial media used to maintain a stable fly colony, and 2.) Compare the microbial communities of stable fly larvae with their developmental substrates.

Materials and Methods

Collection of Substrate and Larvae

The main objectives of this investigation were to characterize and compare the microbial communities of third-instar stable fly larvae

with their developmental substrates. To do this, larvae and substrate were collected under three conditions:

Field Substrate, Field-Collected Larvae

Third-instar larvae were collected from calf bedding at a local dairy (Firth, NE) in late July 2013. The calf pen in which the bedding was located housed six calves <1 yr old and measured ~6.3 m long by 3 m wide. The bedding consisted of corn stalks, manure, urine, and sand and had been in use for 3 wk before being sampled. Substrate from the upper 10 cm was collected in a 50-ml conical screw cap tube, all larvae and pupae removed, and frozen at −80 °C for 2 wk until processed for DNA extraction. Approximately 2 h elapsed between sample collection and freezing. Ten third-instar larvae were also collected from the bedding, rinsed in sterile distilled water, and frozen at −80 °C for 2 wk (subsequently referred to as "natural larvae").

In natural substrates, it is common to find aggregates of stable fly larvae in some locations and none in others. The cause of this behavior is unknown. Therefore, a subobjective was to compare the microbial communities in substrates where stable fly larvae were absent and present. From the same pen, a sample of calf bedding in which larvae were absent and a sample in which they were present (subsequently referred to as "natural-positive" and "natural-negative," respectively) were used for DNA extraction and to analyze physiochemical parameters including water content, electrical conductivity (EC), nitrate and ammonium concentrations, and total carbon (C) and nitrogen (N). Moisture was calculated by weighing 50 g of calf bedding, drying at 100 °C for 48 h, and weighing the dried sample. Moisture of the sample was calculated relative to dry weight, $\text{Moist} = (W_{wet} - W_{dry})/W_{dry}$ (CIMMYT 2013). Laboratory $EC_{1:1}$ was determined in 1:1 water:substrate slurry using a conductivity meter for $EC_{1:1}$ and a glass electrode for pH (Smith and Doran 1996). Nitrate-N was determined using the Cd reduction method (Mulvaney 1996). Inorganic N in 1 *M* KCl extracts was measured colorimetrically using a flow injection ion analyzer (Zellweger Analytics, Lachat Instruments Div., Milwaukee, WI). Total C (g kg^{-1}) and total N (mg kg^{-1}) were measured by dry combustion (EA1112 Flash NC Elemental analyzer, Thermo Finnegan Scientific Inc., Waltham, MA) using air-dried, ground samples.

Field Substrate, Colony Larvae

Calf bedding was collected from the same source and on the same date previously described and brought into the laboratory where it was held at room temperature (~25 °C) for 2 wk, or until the bedding was ~5 wk old. During this time, larvae that were naturally present in the substrate emerged and were removed. Stable fly eggs were obtained from a laboratory colony initiated in 2008 and maintained at the United States Department of Agriculture, Agricultural Research Service, Agroecosystem Management Research Unit, Lincoln, NE, following the methods of Berkebile et al. 2009. Four hours after oviposition, eggs were collected, rinsed, and inoculated into the natural substrate. Larvae developed at room temperature (~25 °C) and, prior to the wandering stage, 10 third-instars were collected, rinsed in sterile distilled water, and frozen at −80 °C until further processing (subsequently referred to as "field larvae"). At this time, a sample of the substrate was also collected and frozen (subsequently referred to as "field substrate").

Lab Substrate, Colony Larvae

Stable fly eggs were collected from the same stable fly colony described previously and were inoculated into larval media consisting of wheat bran, fish meal, wood chips, and water (Berkebile et al. 2009). Larvae developed at room temperature (~25 °C). Substrate and third-instars were collected prior to the wandering stage and frozen at −80 °C (subsequently referred to as "laboratory substrate" and "laboratory larvae").

DNA Extraction, Purification, Amplification, and Sequencing

DNA extraction was performed using the MoBio PowerSoil kit (Carlsbad, CA) according to the manufacturer's guidelines. Approximately 0.25 g of substrate was homogenized and used for DNA extraction. Each larval sample for DNA isolation consisted of a pool of 10 larvae. The bacterial 16S ribosomal RNA gene variable regions 1–3 (Dowd et al. 2011) and the 18S fungal small subunit gene (Handl et al. 2011) from each sample were amplified and the product was purified using Agencourt Ampure beads (Agencourt Bioscience Corporation, MA). HotStarTaq Plus Master Mix (Qiagen, Valencia, CA) was used in a single-step 30 cycle reaction consisting of: 94 °C for 3 min; 28 cycles of 94 °C for 30 s, 53 °C for 40 s, and 72 °C for 60 s; 72 °C for 5 min. Both 16S and 18S rRNA PCRs were performed on negative controls to check for contaminants. No products were obtained, suggesting that the products obtained from the insect samples were largely contaminant-free. Primers and custom barcodes are listed in Table 1 (Dowd et al. 2008). The 16S and 18S amplicons were barcoded and combined into two separate library pools, one containing the 16S libraries and the second containing the 18S libraries. The 16S and 18S amplicon pools were each sequenced on 1/4 plate using a Roche 454 FLX titanium instrument following the manufacturer's guidelines.

rRNA Analysis

Both 16S and 18S sequences were assigned to operational taxonomic units (OTUs) using Mothur v.1.35.1 (Schloss et al. 2011). Samples were demultiplexed, barcodes and primer sequences were removed, and low quality reads were filtered from the dataset. Flowgrams were trimmed to 450-550 bases and denoised using the shhh.flows command in MOTHUR, which is mothur's implementation of the PyroNoise algorithm (Quince et al. 2009). Reads were dereplicated using the unique.seqs command and the pre.cluster command was used to remove reads that differed by 2 bases or less from the dataset, which are likely attributed to pyrosequencing errors. Chimeric reads were identified using the MOTHUR implementation of UCHIME using the reference='self' option. 16S and 18S reads were aligned to the 16S SILVA nr_v123 reference and 18S

Table 1. Primers and barcodes used for 454-pyrosequencing of third-instar stable fly larvae and their developmental substrates

Function	Sequence (5′-3′)
16S forward primer (27F)	AGRGTTTGATCMTGGCTCAG
16S reverse primer (519R)	GTNTTACNGCGGCKGCTG
Eukaryotic 18S forward primer	TGGAGGGCAAGTCTGGTG
Eukaryotic 18S reverse primer	TCGGCATAGTTTATGGTTAAG
454 adapter	AGRGTTTGATCMTGGCTCAG
Barcode 1 lab substrate	ACTGCAGT
Barcode 2 field substrate	ACTGCTCT
Barcode 3 lab larvae	ACTGGACT
Barcode 4 field larvae	ACTGGAGA
Barcode 5 natural positive	ACTGGTCA
Barcode 6 natural negative	ACTGGTGT
Barcode 7 natural larvae	ACTGTCAG

Silva nr_V119 reference, respectively. Reads were assigned to OTUs at 97% similarity using the average neighbor algorithm, which uses a weighted average of the distances between two sequences to determine whether or not they belong to the same OUT. Further, 16S and 18S OTUs were taxonomically classified using the wang approach of the classify.seqs command in mothur, which calculates the probability that the amplicon belongs to each taxonomic rank (phylum, class, order, etc) by its kmer composition and assigns the amplicon to the taxonomy with the highest probability. 16S sequences were taxonomically classified by comparison to Ribosomal Database Project (using trainset9_032012, version 9) (Wang et al. 2007) while fungal 18S OTUs were classified by a BLASTN comparison to the non-redundant nucleotide database. For 18S, the top 100 highest scoring BLAST alignments with evalues ≤1E-08 were retained and used for taxonomic classification. An 80% confidence value was required for all taxonomic classifications and mitochondrial 16S rRNA and genomic 18S rRNA sequences derived from host were eliminated from the analysis. Libraries from each community were normalized by randomly subsampling (without replacement) the same number of amplicon reads ($n = 3{,}216$) to prevent biases from sequencing yields from driving similarities and differences between communities. In order to assess shared species richness and diversity, mothur tools were used to generate rank abundance plots, Chao, Ace, Jacknife -richness, and Shannon-diversity calculations. Distance matrices and heatmaps were generated in R (version 3.0.2) using the vegan, cluster, and gplot packages (Oksanen 2015, Maechler et al. 2016). NMDS analyses were performed using the "metaMDS" command in the "vegan package" of the R statistical computing environment (R Core Team 2016).

Availability of Sequencing Data

Raw 454 sff files containing the 16S and 18S amplicons used in this study have been deposited in NCBI's Sequence Read Archive (SRA) under the accession numbers SRX2071047-SRX2071060 associated with BioProject PRJNA341400.

Microscopy

Images of microflora in stable fly larvae and adults were taken with phase-contrast, confocal, and scanning electron microscopy. Third-instar larvae collected from natural-positive substrate were rinsed in sterile, distilled water and dissected in sterile phosphate-buffered saline (PBS). The digestive system was removed and viewed under phase-contrast and confocal microscopy. Smear mounts of the digestive tract were viewed under a 40X phase-contrast 40/0.65, 160/0.17, Ph3 DL objective on a Nikon Optiphot Phase Contrast microscope (Nikon Corp., Tokyo, Japan). Images were taken with a Nikon D7000 digital camera using a VariMag DSLR adaptor (VariMag, Cape Coral, FL). Smear mounts were also viewed with an Olympus FV500 inverted (Olympus IX-81) confocal microscope (Olympus Corp., Tokyo, Japan). Adult female stable flies were aspirated from resting sites, rinsed in sterile distilled water, dissected in PBS, and the digestive system removed and placed in a 1.5-ml centrifuge tube containing 2.5% glutaraldehyde for at least 24 h at 5 °C. The digestive system was then serially dehydrated in 30%, 50%, 70%, 80%, and 90% ethanol for 15 min/step, followed by two 30 min submersions in 100% ethanol. Specimens were mounted on aluminum studs and sputter coated with gold for examination with a field-emission (Hitachi S-4700) scanning electron microscope (Hitachi Corp., Tokyo, Japan). Confocal and scanning electron images were taken at the Microscopy Core Research Facility, University of Nebraska, Lincoln.

Results

Physiochemical Profile of Natural Substrate

The calf bedding was a moist, slightly alkaline substrate with C:N ratios between 17:1 and 20:1 and relatively high concentrations of ammonium (Table 2).

Composition of Bacterial Communities Associated With Larvae and Substrates

A total of 78,418 high-quality reads from seven stable fly larvae and substrate samples were analyzed using the V1–V3 regions of the 16S rRNA genes (Table 3). After removing low quality reads, denoising flowgrams, and removing redundant reads, 9,410 unique sequences remained. Of these, 2,207 were flagged as potential chimeras and were removed from the analysis while 66 OTUs were eliminated from the analysis because they were not of microbial origin.

A total of 1,431 bacterial OTUs were detected across all stable fly and food substrate samples, ranging from a low of 51 OTUs in the natural larvae sample to 725 OTUs in the field substrate. Among all of the samples analyzed, 44% of the reads were derived from the top 10 most abundant OTUs, with 90% of the reads originating from 305 OTUs. Considering larvae and substrates as groups, 75% of the reads derived from larvae were represented by the top 10 OTUs and 90% of the reads were derived from 30 OTUs. In contrast, only 40% of the reads derived from substrates were represented by the top 10 OTUs, with 270 OTUs required for 90% sequence coverage. Rank abundance plots (Fig. 1) indicated that the bacterial communities from substrates were more diverse in comparison to the larvae, particularly for the natural substrates. The higher diversity of the substrate communities compared to those derived from larvae was confirmed using Chao, Ace, Jackknife and Simpson indices (Table 4). In addition, the Shannon index, which acts as a proxy for community diversity, was higher in the substrates compared to the larvae while the Simpson index, which represents the probability that two individuals selected randomly from the community belong to the same OTU, was significantly lower in the substrates (Table 4). These findings suggest that the communities sampled from the substrates are more complex in comparison to those associated with the larvae.

There were eight OTUs that were found in common to all three larval samples. Of those, five OTUs were found exclusively

Table 2. Physiochemical properties of calf bedding with (positive) and without (negative) stable fly larvae

Larvae	Moisture	EC (mS)	pH	NO_3 (μg/g)	NH_4 (μg/g)	N (%)	C (%)	C:N
Positive	3.43	3.58	8.14	18.41	1116.01	1.62	31.89	19.7:1
Negative	2.91	7.72	7.98	42.72	3166.79	2.05	35.63	17.4:1

Table 3. Bacterial raw and filtered read counts from stable fly larvae and their developmental substrates

Sample	Raw reads	Filtered reads
Field larvae	9,470	8,055
Field substrate	12,906	14,313
Laboratory larvae	7,334	4,562
Laboratory substrate	22,105	19,051
Natural larvae	17,089	12,294
Natural substrate-positive	3,843	3,216
Natural substrate-negative	19,440	16,990

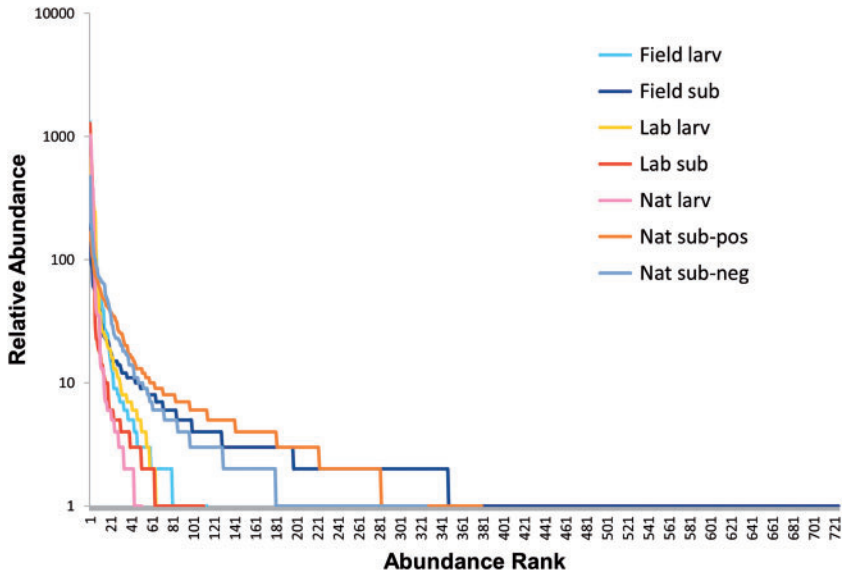

Fig. 1. 16S rank abundance plots of stable fly larvae and their developmental substrates.

Table 4. Bacterial richness and diversity in stable fly larvae and their developmental substrates

Measure	Field larvae	Field substrate	Laboratory larvae	Laboratory substrate	Natural larvae	Natural substrate positive	Natural substrate negative
OTUs	115	725	81	112	52	380	326
Chao	142 (127–177)	1197 (1085–1344)	100 (87–143)	196 (151–291)	55 (53–67)	458 (427–510)	539 (457–641)
Ace	149 (133–1181)	1689 (1556–1844)	92 (85–111)	209 (175–259)	57 (53–69)	449 (426–485)	671 (600–761)
Jackknife	150 (133—167)	1489 (1335–1643)	100 (87–112)	233 (180–286)	61 (53–69)	484 (454–514)	764 (589–940)
Shannon	2.62 (2.54–2.67)	4.96 (4.88–5.04)	2.79 (2.73–2.82)	2.07 (2.01–2.13)	1.97 (1.93–2.02)	4.98 (4.93–5.03)	4.23 (4.17–4.30)
Simpson	0.19 (0.18–0.20)	0.06 (0.05–0.06)	0.12 (0.11–0.12)	0.24 (0.23–0.25)	0.22 (0.21–0.22)	0.014 (0.013–0.015)	0.038 (0.035–0.041)

Parentheticals represent the upper and lower boundaries of the 95% confidence intervals. Chao, Ace, and Jackknife estimate the number of OTUs truly present in each community while the Simpson's index represents the probability that two individuals selected at random belong to the same OTU while the Shannon index quantifies entropy, or the degree of difficulty in predicting which OTU an individual belongs to.

associated with the larvae and were not detected in the substrates. There were also eight OTUs common to all three substrate samples, with seven being found exclusively in the substrate (Table 5). The larval communities contained a lower number of singleton OTUs (defined as OTUs represented by a single read), compared to the substrate communities. Of the 1,431 total OTUs detected across all samples 603 (42%) were singletons, with 55 (4%) of the singletons derived from the larval communities compared to 548 (38%) derived from the substrates, suggesting that the microbial community of the substrates is more rich in comparison to larval communities. The natural-positive substrate had 326 OTUs with 146/326 (45%) occurring as singletons, while the natural-negative substrate had 379 OTUs with 98/379 (25%) singletons.

At the phylum level, the larval communities had higher relative abundances of reads assigned to Firmicutes and lower relative abundances of Bacteroidetes compared to their respective feeding substrates (Fig. 2). At the class level, the larval communities were represented by 21 different bacterial classes, while the substrate communities were represented by 33 bacterial classes. Field, laboratory, and natural larvae were represented by 18, 15, and 17 bacterial classes, respectively. Unlike the larval communities that were comprised of similar numbers of bacterial classes, the numbers of bacterial classes detected across each substrate were more variable with 29, 10, 14, and 17 classes detected in field, laboratory, natural-positive, and natural-negative substrates, respectively. The community from the artificial laboratory media was the least taxonomically diverse, with only 10 classes represented in the subsample, while the community from the field substrate was the most taxonomically diverse with 29 classes represented. For the larval communities, 94% of the sequences are represented in the top 10 classes (as measured by abundance). For the substrates, 99% of the sequences are represented in the top 10 classes.

Taking a closer look at the taxonomic assignments within the Firmicutes, it is clear that this phylum is overrepresented in the larval communities compared to the substrate communities. Specifically, the most dominant class within this phylum was Erysipelotricha, represented by 10 OTUs (Fig. 3), with the primary contributors being four OTUs assigned to the genus *Erysipelothrix*. Within the phylum Bacteroidetes, the classes Bacteroidia, Flavobacteria, and Sphingobacteria account for the majority of the differences observed between the larvae and substrates. At the deeper taxonomic levels, the only genus consistently higher in abundance (3 OTUs) in the larval communities compared to substrates is *Dysgonomonas* (Fig. 3). In addition to differences between larval

Table 5. 16S OTUs in all three larval communities or all three substrate communities

OTU	Phylum	Genus	Total sequences	No. larvae w/OTU	No. substrates w/OTU
14[a]	Proteobacteria (ϒ)	*Pseudomonas*	769	3	0
83[a]	Proteobacteria (β)	Unclassified *Comamonas*	118	3	0
112[a]	Sphingobacteria	*Lacibacter*	64	3	0
63[a]	Proteobacteria (β)	*Castellaniella*	49	3	0
85[a]	Firmicutes	Unclassified Ruminococcaceae	8	3	0
15	Planctomycetes	*Blastopirellula*	379	3	1
1	Actinobacteria	*Arthrobacter*	2592	3	1
3	Bacteroidetes	Unclassified Flavobacteriaceae	1383	3	2
7	Firmicutes	Unclassifed Clostridiales	280	1	3
5[a]	Proteobacteria (ϒ)	*Ignatzschineria*	628	0	3
19[a]	Bacteroidetes	Unclassifed Bacteroidetes	181	0	3
13[a]	Proteobacteria (ϒ)	Unclassifed Xanthomonaceae	153	0	3
24[a]	Bacteroidetes	*Myroides*	142	0	3
6[a]	Actinobacteria	*Microbacterium*	120	0	3
4[a]	Proteobacteria (ϒ)	*Pseudomonas*	768	0	3
28[a]	Proteobacteria (ϒ)	*Providencia*	187	0	3

[a] OTU is found exclusively in larvae or substrate.

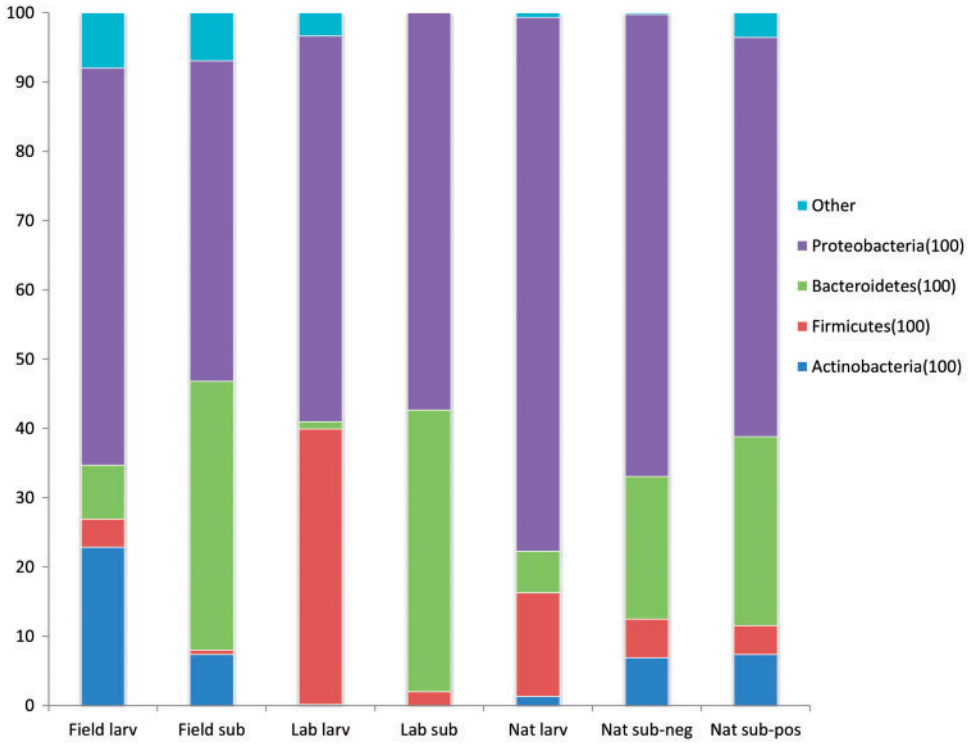

Fig. 2. Phylum-level 16S OTU assignments.

and substrate communities, there are also differences in the abundance of Bacteroides between natural-positive and natural-negative substrates. Specifically, *Dysgonomonas* and *Proteiniphilum* are more abundant in the natural-positive compared to the natural-negative substrate.

Class-level taxonomic assignments within phylum Proteobacteria revealed that larval communities have higher proportions of gammaproteobacteria compared to their respective substrates and a lower proportion of alphaproteobacteria (Fig. 3). The field and natural larvae also had small numbers of reads derived from epsilonproteobacteria that were not observed in their corresponding substrate. Within the gammaprotobacteria, the primary difference between the larval and the substrate communities could be attributed to assignments in the genus *Ignatzschineria*. Larval communities had a combined total of 1,375 reads derived from the genus *Ignatzschinera*, and substrates had a combined total of only 12 reads assigned to this genus. Members of this genus were absent in both the field and natural-negative substrates. The lower proportion of alphaproteobacteria in larval communities is primarily due to sequences derived from field and natural larvae. For example, of the four genera that were more frequently found in the substrates compared to the larval communities, *Paracoccus* and *Sphingopyxix* were found exclusively in the field and natural substrates, *Devosia* was found primarily in both the field and natural substrates, with a single read detected in the field larvae, and *Brevundimonas* was found in all three substrates, with only seven reads assigned to this

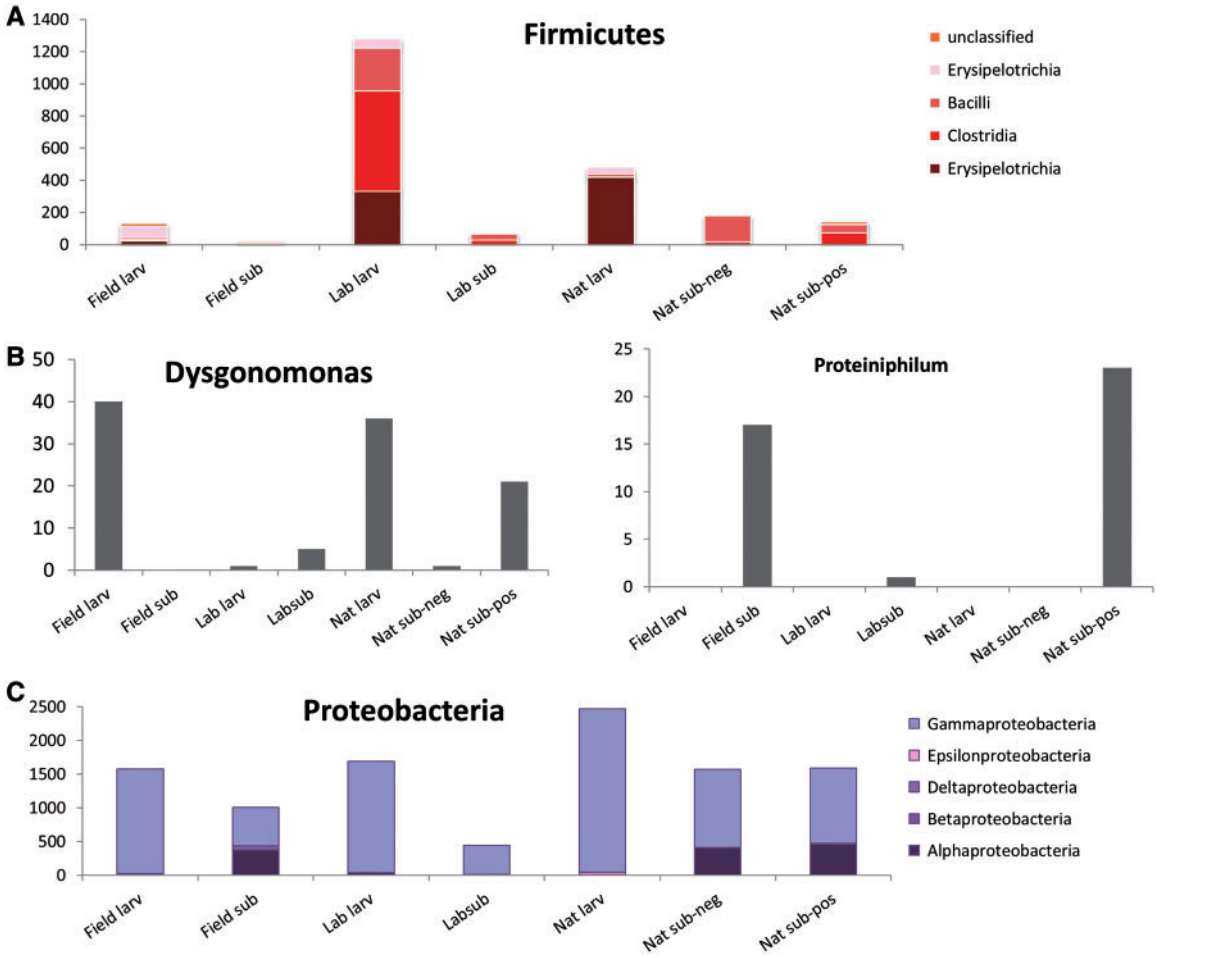

Fig. 3. Class-level 16S OTU assignments. (**A**) Firmicutes. (**B**) Bacteroidetes. (**C**) Proteobacteria.

genus in the laboratory larvae. The lower proportion of betaproteobacteria in the larvae can be attributed to differences in the order Burkholderiales; however, no patterns were observed below this taxonomic level. The small number of epsilonproteobacteria observed exclusively in the field and natural larvae belong primarily to the genus *Campylobacter*.

Nonmetric multidimensional scaling (NMDS) was used to determine whether there were compositional differences between the larval communities and their respective substrates (Fig. 4). Larval bacterial communities are clearly distinct from their developmental substrates. However, the NMDS plot also suggests that substrates can influence the composition of the gut microbial communities to a certain degree due to the relative proximity of each larval sample to its food sample. Heatmaps and dendrograms compiled from a Bray-Curtis dissimilarly matrix support the similarities between the larvae and their food substrates, but clearly show that the larvae are more similar to one another than they are to the substrate on which they are fed (Fig. 5).

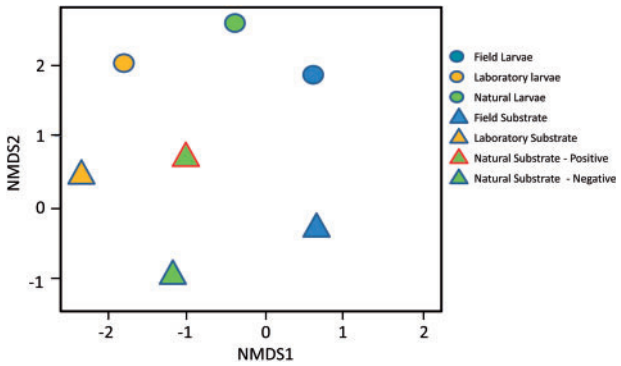

Fig. 4. Nonmetric multidimensional scaling (NMDS) of 16S amplicons. Larval communities (circles) are distinct from, but not independent of, substrate communities (triangles).

Composition of 18S SSU Communities Associated With Larvae and Substrates

A total of 78,841 high-quality sequences from six stable fly larval and substrate samples were analyzed using the 18S small subunit (ssu) rRNA genes (Table 6). The seventh sample (laboratory larvae) was removed due to a lack of 18S microbial reads obtained from this sample. After removing low quality reads, denoising flowgrams, and removing redundant reads, 4,601 unique sequences remained. Of these, 1,803 were flagged as potential chimeras and were removed from the analysis, and 53 were eliminated from the analysis because they were not of microbial origin. There were 29 sequences remaining in laboratory larvae following chimera analysis, and all were eliminated from the analysis following the removal of chloroplast, mitochondria, and archaeal lineages. Subsampling for alpha diversity metrics was performed using a subsample size of 6,019.

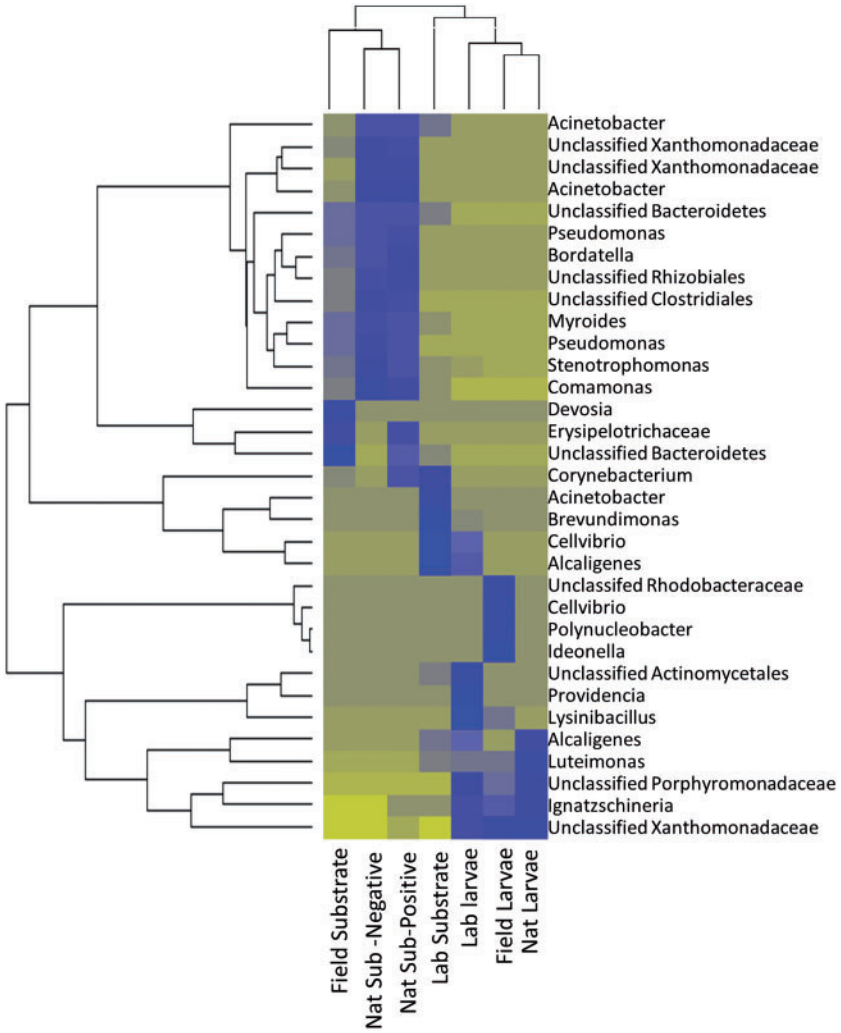

Fig. 5. Larvae have bacterial communities that are distinct from the substrates in which they developed.

Table 6. 18S raw and filtered read counts from stable fly larvae and their developmental substrates

Sample	Raw reads	Filtered reads
Field larvae	6488	6019
Field substrate	22241	18840
Laboratory larvae	3036	0
Laboratory substrate	11287	6384
Natural larvae	12095	11109
Natural substrate-positive	8577	6975
Natural substrate-negative	8282	6700

There were a total of 506 18S OTUs in the six samples. Of these, 395 represented OTUs unique to a single sample, and 191 were singletons. The top 10 OTUs (measured by relative abundance of reads among all six samples) contained 58% of the sequences, and the top 37 OTUs contained 90% of the sequences. The most abundant kingdom-level OTU assignment was to the monophyletic fungi/metazoan Opisthokonts group. This group accounted for 40% of all sequences, and was found in all six samples. The SAR supergroup, representing Stramenophiles, Alveolates, and Rhizaria, were also present in all six samples. A single unclassified protest of the superphylum Alveolata was represented by 3, 88, and 41 sequences in substrates from field, natural-negative, and natural positive, respectively, and was not detected in the corresponding field or natural larvae. Otherwise, no patterns were observed at the kingdom level distinguishing larvae from substrates, or natural-positive from natural-negative substrates. At the phylum level, the top 10 most abundant OTUs belonged to five groups, and included fungal members of both both asco- and basidomycota (Table 7). Rank abundance plots of the 18S data revealed that eukaryotic microbial communities from the substrates were more diverse in comparison to the larvae, displaying both higher richness and higher evenness values (Fig. 6). The higher diversity of the substrate communities compared to those derived from larvae was confirmed using Chao, Ace, Jackknife and Simpson indices (Table 8).

Microscopic surveillance validated the presence of an unclassified Alveolata that appeared to be highly abundant in natural larvae (Table 7). Sporozoites (Fig. 7) and trophozoites (Fig. 8) of an unknown gregarine (phylum Aplicomplexa, class Conoidasida) were tentatively identified in stable fly larvae and adult females, respectively. Sporozoites were located in the larval midgut and Malpighian tubules. Trophozoites were seen on the haemocoel side of the midgut in an adult female stable fly.

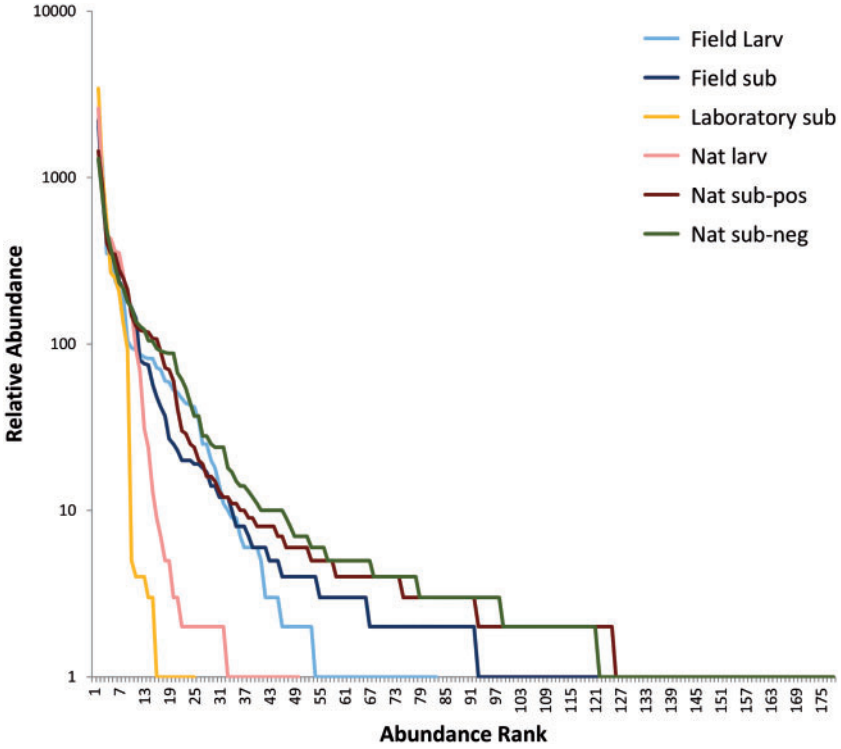

Fig. 6. 18S rank abundance plots of stable fly larvae and their developmental substrates.

Table 7. Top 10 18S OTUs in stable fly larvae and their developmental substrates

OTU	Taxonomy	Field larvae	Field substrate	Lab substrate	Natural larvae	Natural substrate positive	Natural substrate negative
02	Unclassified Alveolata	0	42	0	2612	1302	350
04	Ascomycota	0	0	3431	0	0	0
06	Unclassified Chrysophyceae	2033	416	0	0	232	250
07	Spirotrichea	0	2207	206	0	2	4
01	Ascomycota	82	75	0	0	374	1444
03	Ascomycota	0	331	0	0	292	902
14	Unclassified Chrysophyceae	351	76	0	0	478	347
15	Unclassified Chrysophyceae	1211	0	0	0	0	0
11	Unclassified Eukaryota	0	0	0	907	179	16
20	Basidiomycota	6	23	1057	0	0	0

Table 8. Fungal richness and diversity in stable fly larvae and their developmental substrates

Measure	Field larvae	Field substrate	Laboratory substrate	Natural larvae	Natural substrate positive	Natural substrate negative
OTUs	82	266	24	60	191	183
Chao	130 (101–205)	396 (343–486)	42 (28–105)	75 (65–107)	244 (219–294)	255 (220–324)
Ace	195 (156–256)	389 (347–451)	35 (27–67)	79 (68–107)	245 (223–283)	241 (217–282)
Jackknife	139 (109–169)	490 (409–571)	44 (27–60)	78 (66–90)	254 (231–277)	322 (258–386)
Shannon	2.52 (2.48–2.56)	2.65 (2.62–2.68)	1.43 (1.40–1.46)	1.98 (1.95–2.00)	3.07 (3.03–3.11)	3.22 (3.18–3.26)
Simpson	0.17 (0.16–0.17)	0.17 (0.16–0.17)	0.37 (0.36–0.38)	0.23 (0.23–0.24)	0.10 (0.10–0.10)	0.09 (0.08–0.09)

Parentheticals represent the upper and lower boundaries of the 95% confidence intervals.

Discussion

The microbial community of the third-instar stable fly larvae is unambiguously distinct from the microbial community of the supporting substrate, with bacterial communities from larvae reared on different substrates more similar to each other than to the communities from their individual supporting substrates. Both larvae-specific and substrate-specific OTUs were identified in this particular sample set, as well as OTUs that were clearly more abundant in natural-positive compared to natural-negative substrates.

The microbial communities in third-instar stable flies are less diverse than the environments in which they developed and contain higher abundances of certain taxonomic groups and OTUs. This could be explained by selective host–microbe interactions or larval

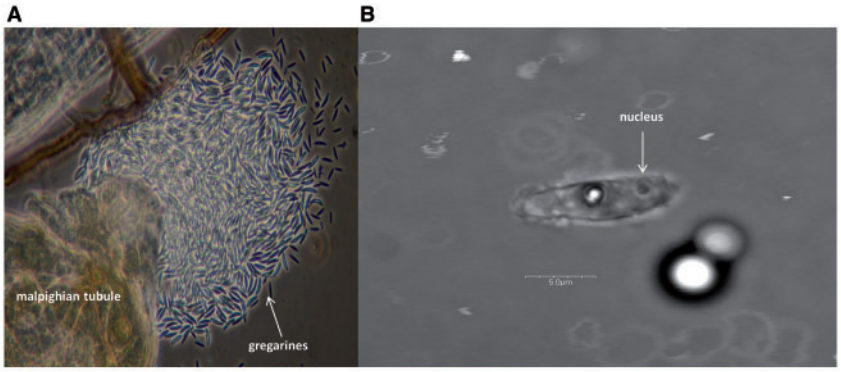

Fig. 7. Phase-contrast (**A**) and confocal (**B**) micrographs of gregarine sporozoites in larval stable flies.

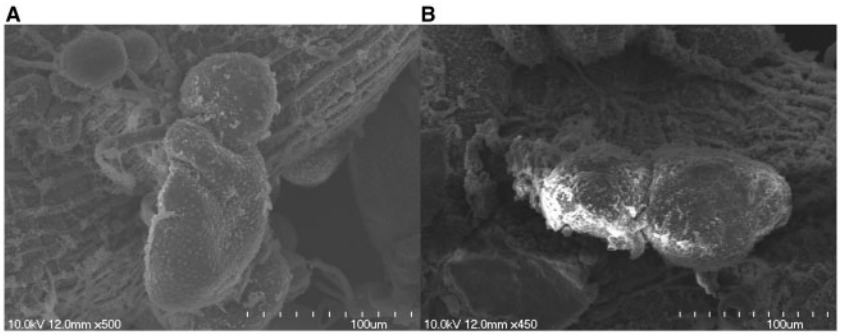

Fig. 8. Scanning electron micrograph of gregarine trophozoites (**A** and **B**) on the haemocoel side of the midgut in an adult female stable fly.

gut physiology. Results from this investigation indicate that while the microbial makeup of developmental substrates has some influence on stable fly bacterial communities, the internal physiology of third-instar stable flies likely has a greater impact on microbial community composition. As with other dipteran larvae, stable flies are equipped with numerous olfactory and gustatory sensillae to interact with their environment (Friesen et al. 2015), which could potential play roles in substrate selection and microbial acquisition from the environment. For example, larval house flies are attracted to ammonium, which is also a common attractant for blood-feeding flies (Hribar et al. 1992). Ammonium is also the main volatile associated with many bacterial cultures and can influence stable fly oviposition behavior (Romero et al. 2006). Given that microbial communities and their metabolites are heterogenous within larval media, it is not unreasonable to predict non-random behavior from stable fly larvae within their environments. In other words, selective grazing on regions of the substrate containing certain microbial communities may explain why some OTUs associated with the substrate are retained in the gut while others were not found.

Additionally, and perhaps more significantly, internal physiology likely influences enrichment and retention of microflora in stable flies, as has been observed for other insects. House fly larvae, which have a gut morphology similar to larval stable flies, employ several digestive mechanisms including a pH gradient and enzymes such as lysozyme and cathepsin D-like proteinase (Espinoza-Fuentes and Terra 1987). In larval house flies, the pH in the fore- and hind-midguts is 6.1 and 6.8, respectively, while the mid-midgut has a pH of 3.1 (Terra et al. 1988). Bacteria appear to be primarily killed in the mid-midgut while the nutrients from lysed bacterial cells are absorbed in the hind-midgut (Lemos and Terra 1991). Bacteria that colonize stable fly guts may also secrete antibacterial products to prevent competition from other bacterial species (Greenberg and Klowden 1972, Erdmann 1987). Oxygen levels within the larval gut may also have had an effect. Three of the four genera that were more abundant in larvae compared to their substrates were facultative anaerobes or microaerophilic, whereas all four of the genera that were more abundant in substrate were aerobic.

Bacterial genera that were more abundant proportionally in larvae compared to their substrates were *Erysipelothrix*, *Dysgonomonas*, *Ignatzschineria* (Gammaproteobacteria), and *Campylobacter* (Epsilonprotobacteria). *Ignatzschineria* is emerging as a common isolate from flies. First described as the genus *Schineria*, these gram-negative, aerobic, rod shaped bacteria were initially isolated from the larvae of myasis-causing flesh flies, *Wohlfahrtia magnifica* (Toth et al. 2001). Subsequently, this genus has been isolated from adult house flies (Gupta et al. 2012) and the blow flies *Lucilia sericata* and *L. cuprina* (Singh et al. 2015). It has also been isolated from the wounds of a 69-year old man found unconscious in a forest in the Loire Valley, France (Le Brun et al. 2015). The same wounds were infested with maggots that were not taxonomically identified but assumed to be *W. magnifica*.

Dysgonomonas isolates have been reported from a variety of samples including human clinical specimens, a microbial fuel cell, and the guts of the two termites *Reticulitermes speratus* and *Macrotermes barneyi* (Hofstad et al. 2000, Almuzara et al. 2009, Kodama et al. 2012, Yang et al. 2014, Pramono et al. 2015). The genus is a coccobacillus-shaped gram-negative, facultative anaerobe. *D. capnocytophagoides* replaced the previously named CDC (Centers for Disease Control and Prevention) group DF-3 (dysgonic fermenter 3) which was characterized as being resistant to penicillins, cephalosporins, aminoglycosides, and ciprofloxacins. In human samples, isolates are typically recovered from immunocompromised patients and have thus been described as a potential opportunistic pathogen. Its impact on animal health is not currently known.

Erysipelothrix are gram-positive, facultative anaerobic, nonsporing rods. The genus includes three species: *E. tonsillarum*, *E. inopinata*, and *E. rhusiopathiae*. The first two species were isolated from the tonsils of healthy pigs and sterile-filtered vegetable peptone broth, respectively (Takahashi et al. 1987, Verbarg et al. 2004). The latter species is an animal pathogen most commonly associated with livestock, specifically swine, and may also cause disease in humans (Brooke and Riley 1999). *E. rhusiopathiae* can manifest itself as erysipelas in calves and may be shed in feces and urine into bedding material. *E. rhusiopathiae* survives for long periods in the environment (up to 5 mo in swine feces, 5 d in drinking water, 14 d in sewage, and 35 d in soil). If this species is able to colonize and survive within stable fly larvae, this insect could transport this pathogen into new environments, thereby increasing the risk of human and cattle disease. Clearly, further research is warranted to determine which species of *Erysipelothrix* is being detected and to characterize its epidemiology in this setting.

Alphaproteobacteria were more abundant proportionally in the natural and field substrates compared to their larvae. Differences were primarily attributed to *Devosia*, *Brevundimonas*, *Sphingopyxix*, and *Paracoccus*. The only other investigation that has utilized 454-pyrosequencing to characterize the microflora of a stable fly developmental substrate (aged horse manure) also described a relatively high abundance of *Devosia*, *Brevundimonas*, and *Sphingopyxis* (Albuquerque and Zurek 2014). *Devosia* spp. are gram-negative, aerobic, rod-shaped bacteria. Most of the species described originated from forest and agricultural soil samples. The genus is known for its ability to survive in environments contaminated with diesels, hydrocarbon pesticides, and mycotoxins. *Devosia* spp. have been isolated from enriched nitrifying communities (Vanparys et al. 2005), which may be important to stable fly larval development (Friesen et al. 2016a). *Brevundimonas* spp. are motile, gram-negative, aerobic, non-lactose fermenting bacilli that were previously classified as *Pseudomonas* (Segers et al. 1994). Among the traits that differentiate the two genera are the single, short flagellum (Leifson and Hugh 1954) and limited biochemical activity of the brevundimonads. A *Brevundimonas spp*. has been isolated also from house fly pupae and puparia collected from cattle manure (Zurek and Nayduch 2016).

The main genera responsible for differences between the positive and negative field substrates were *Dysgonomonas* and *Proteiniphilum*. In contrast to *Dysgonomonas*, *Proteiniphilum* was more abundant in substrate than in the larvae. *Proteiniphilum* is a gram-negative rod that is an obligate anaerobe. *Proteiniphilum acetatigenes* was the first species described for this genus and was isolated from the sludge in an upflow anaerobic sludge blanket (UASB) reactor (Chen and Dong 2005) that treated brewery wastewater. *Proteiniphilum* has been isolated also from a bioreactor seeded with sewage sludge from a wastewater treatment plant (Maspolim et al. 2015) and waste-activated sludge (Guo et al. 2015). In the latter investigation, it was found that *Proteiniphilum* was more likely to be enriched as lignin and humus content increased. Interestingly, substrates supporting stable fly development on Reunion Island had lignin contents of 4.0–6.2% (Giles et al. 2008). Corn stalks, which were a main constituent of the calf bedding in this investigation, have similar lignin contents ranging from 4.0–7.3% (Saxena and Stotzky 2001, Daud et al. 2013).

The most abundant 18S OTU was assigned to an unclassified Alveolata and was primarily detected in natural larvae and natural-positive and natural-negative substrates. Sporadic surveillance of larval and adult stable fly microflora repeatedly revealed the presence of an organism that was tentatively identified as a gregarine. Gregarines are transmitted when a new host ingests oocysts in the environment. Sporozoites emerge from the oocysts and mature into trophozoites as they attach to and feed on host cells. These parasitic apicomplexans may be detected in the digestive tract, reproductive organs, or coelom of marine, freshwater, or terrestrial invertebrates. Within the Diptera, gregarines have been described in deer flies (Krinsky 1975, Anderson and Magnarelli 1978), crane flies (Ludwig 1946), sand flies (Votypka et al. 2009, Lantanova et al. 2010), and mosquitoes (Chen 1999). Prevalence of gregarines within the Muscamorpha is unknown.

Because physiochemical properties were quantified in only two samples of calf bedding, a comparison of substrates with and without stable fly larvae cannot be made. However, it is worth noting that while the moisture content and pH appear to be similar in calf bedding and hay residue (Friesen et al. 2016b), ammonium levels were higher in calf bedding. The probability of collecting stable fly larvae in hay residue peaked when ammonium levels were between 200–300 ppm, compared to over 1,100 ppm in calf bedding. A more thorough profile of calf bedding is needed to determine if this represents an outlier.

This is the first culture-independent description of the bacterial and eukaryotic microbial community in stable fly larvae and the first description of the eukaryotic microbial community in their developmental substrates. Results from this investigation demonstrate that microbial communities of the larvae are distinct from those of their supporting substrates, and suggest that larval host internal physiology may play a selective role. Results also highlight several areas in need of further research including *Erysipelothrix*–larval interactions, the role of certain Alphaproteobacteria in larval development, the influence of lignin content on microbial community composition and stable fly larval development, and the interactions between eukaryotic microorganisms and stable fly larvae.

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