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The microbiome of fly organs and fly-human microbial transfer during decomposition

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ABSTRACT

During decomposition, flies interact with the remains to lay eggs and acquire nutrients, and in the process, they bring their microbes with them. While it is known that flies have their own unique core microbiome, it is not known if flies associated with human cadavers have a different core microbiome. Differences in the fly microbiome may influence the types of microbes transmitted from the flies to the cadaver, therefore potentially affecting assembly of the human decomposer microbiome. The first purpose of this study was to characterize the microbiome of flies associated with human cadavers by fly organ and season. This is because fly interactions with cadavers vary by season, and because it is likely that external fly organs [i.e., the labellum and tarsi] make more direct contact and are likely involved in increased mechanical transmission with the cadaver than internal organs such as the oocyte. The second purpose of this study was to determine if the fly microbes contribute to the human decomposer microbiome. To accomplish these aims, 10 human cadavers were placed outdoors across three seasons and allowed to decompose. A total of 40 flies that landed on the cadaver were collected and dissected by the labellum, tarsi, and oocyte. In addition to fly collections, samples from the cadavers were collected using a sterile swab at sites including the cheek of the face, inner cheek, bicep, torso, and anus. Overall, it was shown that flies associated with human cadavers have a similar microbiome to flies from previous studies that were not associated with human cadavers. However, there are differences in the microbiome between seasons and fly parts. We also show evidence that flies act as a microbial source to the human decomposer microbiome, which is important for understanding the ecological mechanisms of human cadaver microbial community assembly.

1. Introduction

The decomposition of vertebrate remains is a dynamic process that is partially driven by the actions of microbes [1–5]. As decomposition progresses through successive stages (fresh, early decomposition, advanced decomposition, skeletonization, and decomposition of skeletonized material, as defined by Galloway et al. [6]), the microbial roles change as nutrients derived from cadavers change [7]. Immediately after death and in the fresh stage, enteric microbes are no longer influenced by the host immune system [7]. Those that can survive with little oxygen flourish [7,8], initiating a shift from the individual host microbiome [9] to a decomposer microbiome that is more consistent across cadavers [10]. During this time frame, flies [primarily female] are also interacting with the cadaver, as they are attracted to the nutrients and volatiles produced by microbes [11]. Females will lay eggs in the eyes, nose, and ears, as well as in the hair and in body-body and body-ground interfaces [7,12–14]. Typically, blow flies (Calliphoridae) are the first to colonize a cadaver.

Studies have shown that flies have their own microbiome [15–22]. This fly microbiome has generally been composed of the phyla Proteobacteria, Firmicutes, and Bacteroidetes [15,16], with a small number of species identified within phylum Actinobacteria [19]. Some of the more common bacterial genera associated with flies include *Enterococcus, Proteus, Serratia, Wolbachia, Pseudomonas, Corynebacterium, Providencia*, *Lactobacillus*, *Lactococcus*, *Morganella*, and *Myroides*, although this is not a comprehensive list of every genus found on flies. Additionally, body part specific analyses have been conducted, in which it was found that *Providencia* spp. were

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more abundant on the fly abdomen [15] and *Lactobacillus*, *Proteus*, *Diaphorobacter*, and *Morganella* were dominant in the salivary gland [16]. Another experiment studying the bacterial profiles by organ showed that *Pseudomonas* was a key contributor to all bacterial profiles studied, with notable differences between the digestive tract, salivary gland, and reproductive organs [18]. While these studies were useful for characterizing the fly bacterial microbiome and discovering bacterial differences between body parts, very few studies have been performed in a decomposition environment. Wohlfahrt et al. used decomposed beef liver as an attractant for blow fly species *Lucilia sericata* and *Phormia regina* to characterize the bacterial communities associated with different life stages of fly development [22]. In both fly species, Bacilli and Gammaproteobacteria (classes that are both common to other fly microbiome studies) comprised > 95% of all bacterial classes across all life stages. In the same year, Maleki-Ravasan et al. published a study showing that flies collected using chicken liver baited traps contained bacteria within genera *Enterococcus*, *Myroides*, *Proteus*, *Providencia*, and *Serratia*, all of which are also common genera of the fly microbiome [19]. However, more experiments studying the fly microbiome in the context of human decomposition are needed.

The interaction of flies with carrion and the potential transfer of microbes between hosts in the process makes characterization of the fly microbiome in a human decomposition setting of entomological and forensic importance. There are several studies showing evidence of a mechanical transfer of microbes between flies and carrion, including viruses [23] and bacteria [21,22,24,25]. Transfer can occur several ways, including via physical contact between the fly and the cadaver [22,24], fly defecation and/or regurgitation [21], and oviposition [19]. Flies arrive to lay eggs and feed immediately after death, during which the human decomposer microbiome begins to assemble [10]. Therefore, characterizing the microbiome of flies associated with human cadavers and understanding the mechanical transfer of microbes onto human cadavers is relevant to elucidating the ecological dynamics of the microbiome assembly of human decomposition. Of particular interest are the microbial genera *Ignatzschineria* and *Wohlfahrtiimonas*. These bacteria have been found in association with living humans experiencing myiasis (infestation of living tissues by fly larvae) by calliphorid and sarcophagid flies [26]. In 2015, Hyde et al. recorded *Ignatzschineria indica* on human cadavers after purge that persisted through until the later and drier stages of decomposition [27]. *Ignatzschineria* and *Wohlfahrtiimonas* are bacteria genera of the family Gammaproteobacteria, first recorded from wounds of living humans diagnosed with myiasis [28–30]. *Ignatzschineria* (homotypic synonym *Schineria*) was described based upon the type *Ignatzschineria* larvae by Tóth et al. [31]. Four strains were originally extracted from the first and second larval instars of the sarcophagid fly *Wohlfahrtia magnifica*. The same were also extracted from the gastrointestinal tract of adult sarcophagid flies [28]. In the laboratory, when the contents of guts of larval sarcophagid flies were cultured, *Ignatzschineria* were very common [32,33]. Hyde et al. [27] showed that field research of human cadavers decomposing under natural outdoor conditions have a pattern of bacterial succession occurring where the community structure changes over time. Notably in this study was that after purge and until the cadaver dried out, fly associated Xanthomonadaceae bacteria, specifically *Ignatzschineria*, dominated the microbiome. Their findings showed that *Ignatzschineria* relative abundance was inversely proportional to relative diversity of other bacterial species [27]. Metcalf et al. [4] show patterns in composition of bacterial communities at the level of the family at each stage of decompositions. In their study, Gammaproteobacteria composition increased significantly (seemingly at the expense of other families) during decomposition right before rupture and stayed high all through active decomposition.

The first purpose of this study was to characterize the fly microbiome in a human decomposition environment. This was to understand if fly interactions with human cadavers affected their microbiome. To do this, human cadavers were placed to decompose outdoors, unclothed, aboveground, and in the supine position at the Southeast Texas Applied Forensic Facility (STAFS) in Huntsville, TX. A total of 10 cadavers were placed across three seasons. Seasonal placements were conducted because fly diversity and abundance vary by season [34], which may in turn affect the fly microbiome (although this is only a hypothesis). A total of 40 flies to come into contact with the cadaver were collected (ranging from immediately after placement to hours after placement), and the microbiomes of the labellum (mouth parts), tarsi (leg parts), and oocytes were characterized using 16S ribosomal RNA gene sequencing following the Human Microbiome Project standard protocols [35]. The labellum and tarsi were sampled because they make direct contact with the cadaver, while the oocytes were chosen to preliminarily screen for transovarial transmission, which is how several insect symbionts such as the bacteria *Wolbachia* are known to be transmitted across generations [36]. Since flies interact with human cadavers during the transition of the microbiome from the individual host community to the decomposer community [37], the second purpose of this study was to determine if the fly microbes contribute as a source to the human decomposer microbiome, as well as to investigate if this source contribution differs between seasons. We predicted that part-specific analyses would show that the labellum and tarsi, external fly organs which come into direct contact with the cadavers, would have more similar microbial compositions to each other compared to the oocyte. Furthermore, we also predicted that the labellum and tarsi, which are involved in a higher rate of mechanical transmission of microbes [37], would contribute to the human decomposer microbial community assembly.

2. Materials and methods

2.1. Study site

The Southeast Texas Applied Forensic Science Facility (STAFS, formerly the Applied Anatomical Research Center, AARC) is a willed-body donation facility housed at the Center for Biological Field Studies (CBFS), Sam Houston State University, Huntsville, Texas. It is a research facility with a focus on the study of applications of forensic science of the human body. The facility lies in the Pineywoods ecoregion of Southeast Texas and has a subtropical, humid environment with a moderate covering of pine trees and herbaceous underbrush. The soil is acidic, well-draining, and sandy [38].

2.2. Cadaver placement and monthly temperature calculations

As part of a larger three-year study looking at the ecology of decomposition, 10 human cadavers were placed outdoors over three seasons and allowed to decompose under natural conditions with no clothing and no cage protection [5,27]. Cadavers were not autopsied and were either cooled, frozen, or underwent both before placement. The beta diversity of the cadavers based on whether they were cooled, frozen, or underwent both can be seen in Fig. S1. A summary of cadaver information including age, sex, storage conditions, height, weight, ancestry, and medical history is provided in Table S1. The average monthly temperatures for seasonal placements were calculated by collecting monthly summary data from Weather Underground and averaging both February placement months (2014 and 2015) together to get the overall February average and averaging both April placement months (2014 and 2015) together to get the overall April average. There was only one July placement (2014), and the monthly average for this one month was used. It is worth noting that seasons in subtropical, southeast Texas tend to be warmer than

in other climates. A breakdown of the average monthly weather data is provided in Fig. S2, including average monthly temperatures, average monthly precipitation, and average dew points.

2.3. Fly collections

Once the bodies were placed, the goal was to collect the first 40 flies that were in contact with the cadaver as this would target those flies associated with the earliest stage of decomposition when the microbiome of the cadaver is assembling and would represent possible sources of bacteria. For some bodies (primarily those in the winter season when insect activity is limited), there were not 40 flies to collect. Those in the field were careful to make sure that the flies had landed on the cadaver before collection. The time for collection ranged from immediately after placement to hours after placement. The wide range in time was mainly dependent on time necessary for the body to thaw (affecting fly attraction to the remains), and the outside temperature (affecting fly availability and fly activity). To collect the flies, three different methods were used: collection by hand (gloved), collection by aerial sweep nets, and collection directly into conical tubes (most used as it is the easiest method). The authors recognize that it is possible that the flies that were collected could have easily interacted with other decomposed bodies within the facility. However, since the purpose was only to ensure that flies has indeed landed on a cadaver and had the chance to acquire human decomposer microbes, and the point was not to track microbes from a specific body onto a specific fly (or vice versa), the authors do not believe that this has affected the conclusions of this study. Upon collection, flies were kept in separate sterilized conical tubes, placed in a bag labeled with the body accession number, and frozen until dissection.

2.4. Human sampling

Samples from cadavers were collected at the same time as fly collection using sterile dual-tipped BD SWUBE Applicator (REF 281130) swabs by rubbing the sample site lightly for approximately 30 s over an approximately 2 cm square area. Cadaver sample sites were the bicep, cheek of the face, anus, inner cheek, and torso. First the flies were collected and then the swabs were taken to minimize disruption of the flies and the cadaver.

2.5. Identification of flies

Flies were identified to family using the Field Guide to the Insects of America: North of Mexico [39]. Flies in the family Calliphoridae were identified to genus and species using the Whitworth Key to the Genera and Species of Blow Flies (Diptera: Calliphoridae) of America North of Mexico [40] and flies in other families were identified to genus and species by cross-checking them against a reference collection housed at the Sam Houston State University Museum.

2.6. Dissections

Flies were dissected to obtain all tarsi from one side of their body (the right), the labellum, and the oocytes (if female). All dissections were conducted under sterile conditions using a laminar flow hood. Forceps were sterilized with bleach between each fly dissection. After the tarsi and labellum were isolated but before the oocytes were dissected, the flies were washed in soapy water and rinsed in EtOH. After oocyte dissection, the oocyte surfaces were also washed in soapy water and rinsed in EtOH. All fly organs were placed separately in sterile cryotubes and labeled with fly accession number, fly part, dissection date, and dissector identification. Samples were stored at − 80 °C until they were sent to the Alkek Center for Metagenomics and Microbiome Research at Baylor College of Medicine for sequencing.

2.7. Sample processing and sequencing

The bacterial communities for all fly parts from all species collected and human swab samples were assessed by genetic identification employing high throughput sequencing techniques. Amplification of the 16S ribosomal RNA (rRNA) gene and Illumina sequencing were conducted at the Alkek Center for Metagenomics and Microbiome Research at Baylor College of Medicine following protocols benchmarked as part of the Human Microbiome Project [35]. DNA was extracted from the fly organs or human swabs using the MoBio PowerSoil DNA isolation kit following manufacturer's instructions. Negative controls that were included in the extraction process did not show evidence of amplification following gel electrophoresis and were thus not included in sequencing. 16S rRNA gene sequencing was performed using Illumina MiSeq with barcoded primers targeting the V4 region: GGACTACHVGGGTWTCTAAT and GTGCCAGCMGCCGCGGTAA.

2.8. Data cleaning and analysis

Data cleaning and most analyses were performed using the microbiome analysis package, QIIME2 [41]. All raw data files were imported into QIIME2 version 2021.4 using EMPPairedEndSequences file types, except for one sequencing pool in which only the already merged reads were available and thus the EMPSingleEndSequences file type was used. Each pool was demultiplexed using the demux plugin, and all forward and reverse reads were merged using VSEARCH [42]. Quality filtering using the q-score was performed using the quality-filter plugin [43] and the default parameters. Denoising to create amplicon sequence variants (ASVs) was performed using the denoise-16S method in the deblur [44] plugin with a left trim length of 0 and a right trim length of 250 (i.e., the entire sequences were kept for all pools due to high quality). All feature tables and representative sequence files were merged using the feature-table merge and merge-seqs methods, and all subsequent analyses were performed on merged data.

Taxonomy was assigned using a Naive Bayes classifier trained on SILVA 138 99% OTUs from the 515 F/806 R region of sequences [45–47]. Taxa assigned to chloroplasts and mitochondria were filtered from the dataset. To visualize the observed taxa, the barplot visualizer in the taxa plugin was used. These data were exported to a csv file and imported into R software 4.0.3 [48] for bubble chart visualization (see below for packages used). To create the bubble chart, taxa relative abundances were first calculated within the entire dataset, and the top 50 taxa from this table were visualized. A phylogenetic tree was created using the fragment-insertion plugin [49–52] and the SEPP [53] method using the SILVA 128 SEPP reference database. For simplicity, duplicate seasonal placements (i.e., both winter and both spring placements) were combined for group analyses. Core metric phylogenetic analyses were performed using the insertion tree and with a rarefying depth of 5937 reads per sample as an optimal balance for retaining observed features and samples. From this pipeline, the unweighted UniFrac [54] and weighted UniFrac metrics were used for assessing beta diversity. To compare groups, the permutational multivariate analysis of variance (PERMANOVA) [55] test output from the beta-group-significance visualizer in the diversity plugin was used, with the pseudo-F value used to estimate effect size and the p-value used to assess significance (or q-value for pairwise comparisons). Core taxa within fly and human samples were identified using the core-features visualizer in the feature-table plugin with the default setting of 0.5 as the minimum fraction of samples that a feature must be observed in to be considered a core feature. Differentially abundant taxa

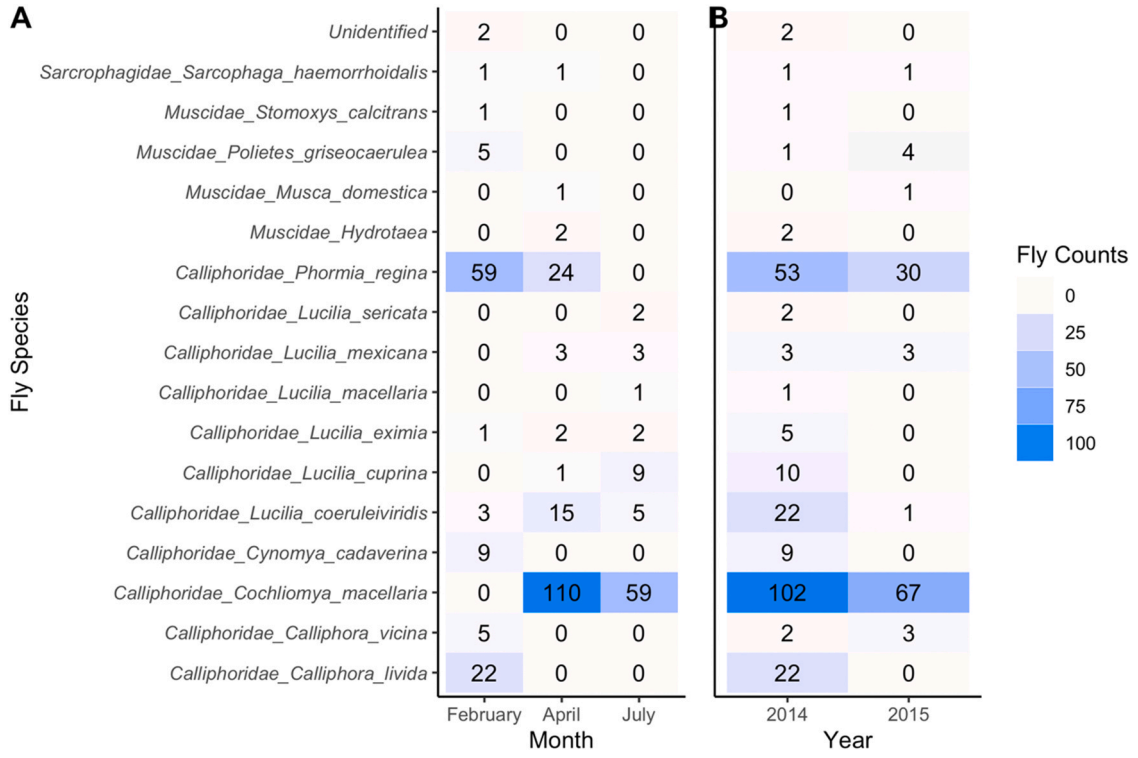

Fig. 1. Summary of fly collections by month (A) and year (B). Taxon names include the family, genus, and species. The "unidentified" fly species is a member of the family Muscidae, and the species of *Hydrotaea* could not be identified but is likely *Hydrotaea aenescens*.

between groups were identified using analysis of composition of microbiomes (ANCOM) [56] in the composition plugin. To do pairwise ANCOM analyses, feature tables containing only two categories within a group (e.g., tarsi and labellum, tarsi and oocyte, labellum and oocyte) were created. ANCOM was applied to each table, and the results between tables were compared. This method was also used for pairwise ANCOM analyses between seasonal placements.

For source tracking, separate feature tables were created for each seasonal placement and each table was exported from QIIME2 as a BIOM 2.1.0 table. These tables were used to generate per season source predictions with the Gibbs function in SourceTracker2 [57] in which fly parts were used as sources and human sample types were used as sinks. Since rarefied BIOM tables were used, source rarefaction depth and sink rarefaction depth were 0 in all cases.

All visualizations were made in R software 4.0.3 [48] using the following packages: ggplot2 [58], reshape and reshape2 [59], ggpubr, qiime2R [60], tidyverse [61], ggpattern, plyr [62], and sf [63].

2.9. Data availability

All data are available in QIITA study 13301 and all analysis and visualization code files are provided at https://github.com/Metcalf-Lab/fly_human_2021_Deel.

3. Results and discussion

3.1. Fly occurrence

The fly species that were collected across the five different placements (February, April, and July 2014, and February and April 2015) are summarized in Fig. 1 by month and year. These data are consistent with the collection times from other years for these species of fly for our geographic region (unpublished data available from Sam Houston State University entomological collection).

3.2. Quality of amplicon sequence data

A total of 16,970,884 reads were generated. Filtering of reads assigned to chloroplast and mitochondria resulted in a total of 16,235,066 reads with a mean frequency per sample of 13,461 and a range of 2–55,152 reads. To normalize, the data were rarefied at 5937 reads per sample as an optimal balance for retaining enough samples and observed features. This retained approximately 74% of all samples (890/1206), with the percent of retained samples for each sample type as follows: 69% fly, 81% bicep, 82% face, 84% fecal, 81% inner cheek, and 84% torso.

3.3. The fly microbiome

The main phyla found in every fly part investigated of every species included in the analyses were Proteobacteria, Firmicutes, Bacteroidetes, and Actinobacteria (Fig. 2). This result agrees with several other studies that observed the fly microbiome [11,15–17,64]. One notable exception is the relatively smaller abundance of Actinobacteria in February (Fig. 2). In this dataset, the top three most relatively abundant taxa that comprise phylum Actinobacteria are within genus *Corynebacterium* (including an unclassified *Corynebacterium*, *Corynebacterium urealyticum*, and *Corynebacterium propinquum*, in decreasing order). Since this genus grows best within a temperature range of 30–37 °C (86–98.6°F) [65], perhaps *Corynebacterium* can't survive within the colder February temperatures.

A total of seven core features were identified using the QIIME2 core-features plugin, and these were (in decreasing order of frequency) genera *Tumebacillus*, *Vagococcus*, *Wolbachia*, *Providencia*, *Pseudomonas*, *Staphylococcus*, and family *Comamonadaceae*. Although *Wohlfahrtiimonas*, a common fly-associated bacterium [66], was found in the fly microbiome (Fig. 2), it was not identified as a core feature in this dataset. *Tumebacillus*, the most relatively abundant core feature, is not commonly associated with flies [11,15–17,64], but it is a gram-positive aerobic organism that has previously been

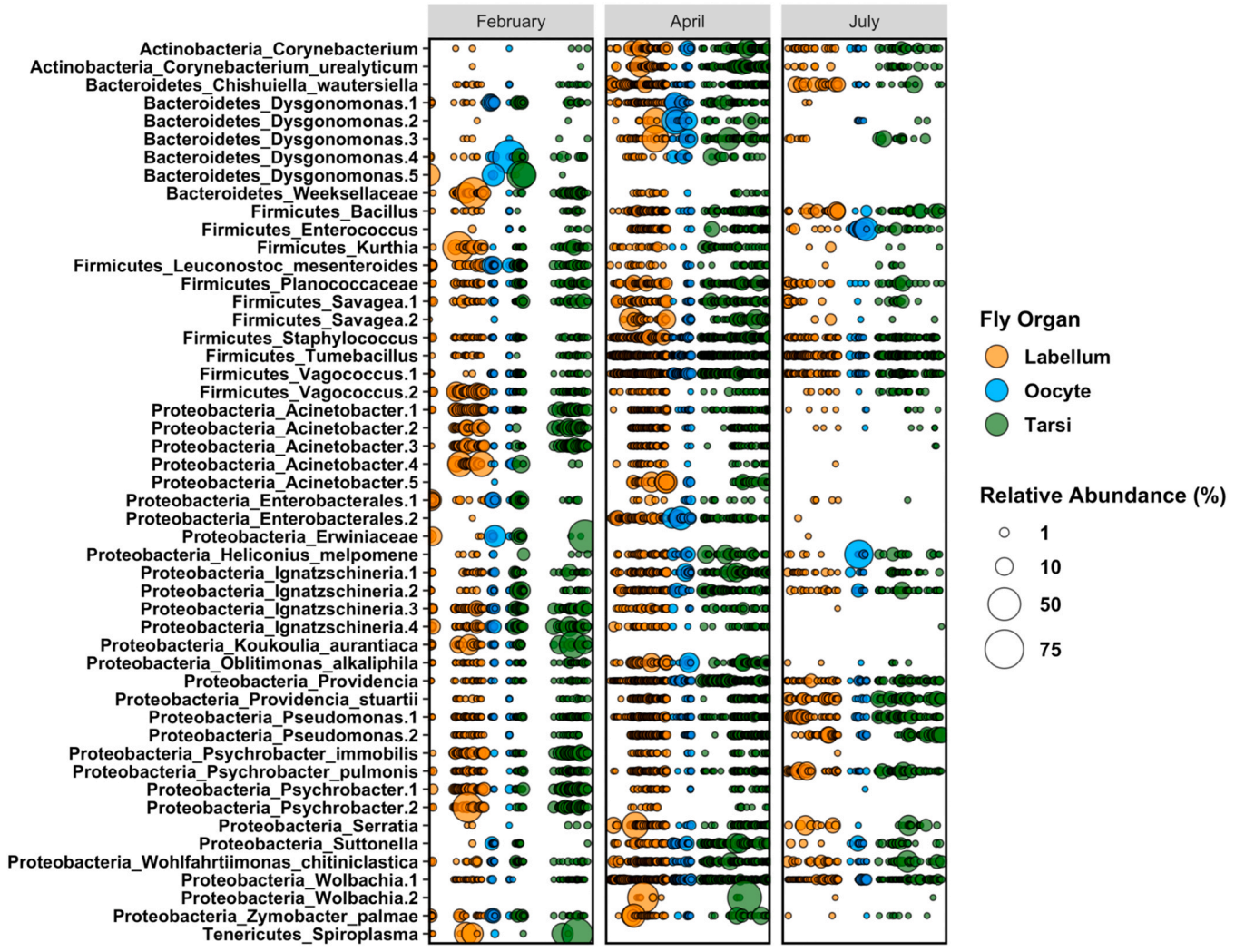

Fig. 2. A bubble chart of the relative abundance of the top 50 fly taxa colored by organ and separated by placement season. Taxa are sorted by phylum, and each taxon name contains the phylum and the taxonomically lowest identifiable name. Each column represents the community of a single fly organ within the indicated season, and each row shows the relative abundance of the specified taxon (with all bubbles in the row adding up to 100%). Taxa with number assignments represent different amplicon sequence variants that were assigned to the same taxon.

found in non-rhizosphere soils [67] and was likely transferred onto the flies from the surrounding outdoor environment. *Vagococcus*, *Providencia*, *Pseudomonas*, and *Staphylococcus* are all known to be present on flies from previous studies [15–17,19,21,22,37,66]. *Wolbachia* is known for its endosymbiotic relationships with arthropods, including reproductive manipulations as well as protection against pathogens [68]. In calliphorid flies, *Wolbachia* is the most abundant and ubiquitous organism for all body parts [15]. In our dataset, *Wolbachia* varied in its presence. For example, *Wolbachia* was present in all fly organs and represented the majority of features in nearly all samples for flies *Lucilia coeruleiviridis*, *Lucilia eximia*, and *Lucilia mexicana*, but for *Phormia regina*, *Wolbachia* presence ranged from dominating all samples from all organs (typically in the April placements) to being low in frequency or undetectable (typically in the February placements). There were also several other fly species including *Calliphora vicina*, *Cynomya cadaverina*, *Calliphora livida*, *Lucilia cuprina*, and *Lucilia sericata* in which *Wolbachia* had little presence. However, it is important to note that many of these *Wolbachia*-lacking species were collected only in one seasonal placement, so it is difficult to determine whether this is a species or seasonal effect. The last core feature, family Comamonadaceae, is a diverse bacterial family that comprises over 100 species in at least 29 genera [69]. To the authors' knowledge, this family has not been well highlighted in fly microbiomes.

Outside of the core features, there was a notable taxon present at a high relative abundance found within the fly microbiome that is typically known to be only human-associated or associated with environments that flies in our environment are not known to interact with. Five different amplicon sequence variants (ASVs) assigned to genus *Dysgonomonas* were identified (Fig. 2). This taxon has been isolated from environments like the human gallbladder [70], abdominal drains [71], and wounds [72]. While it has also been isolated from the gut of a termite [73], it seems more likely that the flies acquired *Dysgonomonas* through their continued interaction with decomposing humans rather than termites, which are not common and have not been recorded in association with decomposing human remains at STAFS. However, it is also possible that *Dysgonomonas* is naturally occurring in the fly microbiome. It is interesting to note that *Dysgonomonas* had a noticeably higher relative abundance in April compared to other seasonal placements, the reason for which requires further investigation. Although this was a surface-level observation, it indicates that flies likely can pick up human-derived bacteria from cadavers after only a few hours of decomposition.

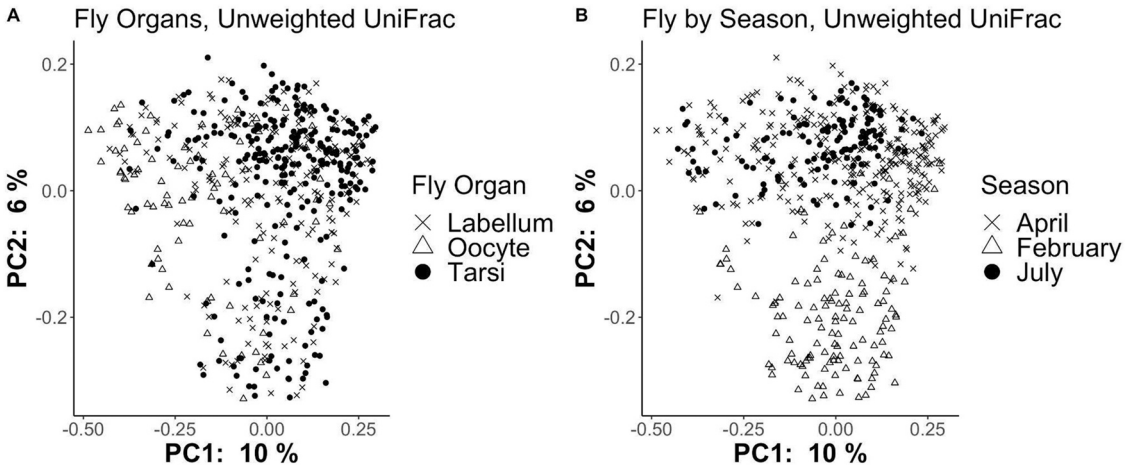

Fig. 3. Unweighted UniFrac beta diversity of fly microbiomes by organ and season. For all unweighted PERMANOVA pairwise comparisons (i.e., all organs compared to all organs and all seasons compared to all seasons, see methods) q = 0.001 (999 permutations). The weighted UniFrac version of this can be seen in Fig. S4.

3.4. How does the fly microbiome compare between the tarsi, oocyte, and labellum?

Pairwise comparisons between the beta diversity of fly parts (Fig. 3A) were significant for all beta group unweighted UniFrac and beta group weighted UniFrac tests (PERMANOVA q = 0.001 for all comparisons, 999 permutations). Comparisons including the oocyte had higher pseudo-F values for both unweighted UniFrac (pseudo-F = 11.89 and pseudo-F = 15.07 compared to the labellum and tarsi, respectively) and weighted UniFrac (pseudo-F = 11.05 and pseudo-F = 11.33 compared to the labellum and tarsi, respectively) metrics compared to labellum versus tarsi comparisons (pseudo-F = 3.05 and pseudo-F = 4.24 for unweighted UniFrac and weighted UniFrac, respectively). These statistics indicate that the bacterial composition of the oocyte is significantly different from the tarsi and labellum. Pairwise comparisons using the ANCOM plugin in QIIME2 identified 14 differentially abundant features between fly parts (Table 1). Notable features included *Chishuiella wautersiella*, which was more frequent in the labellum compared to the tarsi and oocyte, *Ignatzschineria*, which was more abundant in the tarsi compared to the labellum, *Suttonella*, which was more abundant in the tarsi and oocyte compared to the labellum, and several other features that were more abundant in the labellum and tarsi compared to the oocyte such as *Tumebacillus*, *Psychrobacter pulmonis*, and *Pseudomonas* (Table 1). Genus *Chishuiella* is a gram-negative, strictly aerobic bacterium that has been isolated from freshwater [74]. STAFS is located at the Center for Biological Field Studies, a 250-acre land designation. There are two main watersheds in the area, Wynne and Harmon, with smaller tributaries which cross throughout the area, including within the STAFS facility [75]. *Ignatzschineria* is a bacterium that is commonly associated with myiasis, or infection by fly larvae of human tissue though it's exact role in maggot biology is not understood [16,27,29,30,76–81]. While we expected to find this common fly bacterium on both the tarsi and the labellum, it is more common on the tarsi. This may be simply because the surfaces of the tarsi are in contact with cadaver tissues for longer than the labellum and allows for increased transfer of bacteria. Interestingly, *Suttonella* has been found to be associated with human respiratory disease [82]. This may be further evidence of two things. First, flies can pick up human-derived bacteria from cadavers during decomposition. While it has been known for several decades that flies can pick up bacteria from other sources, this knowledge can potentially be extended to include flies picking up bacteria in a human decomposition environment. Second, not only do flies pick up human-derived bacteria, but these bacteria then may become integrated into the fly microbiome. Even when a new bacterium is introduced into an environment, it is possible that the ecological dynamics of the microbiome in the environment do not support the integration of the new bacterium into the microbial community structure. Therefore, it is interesting that the fly microbial community dynamics support integration of bacteria from human cadavers into their microbiome. Our prediction that several features would be more differentially abundant in the labellum and tarsi was confirmed. This is likely due to the increased interaction of these surface organs with the surrounding environment compared to the internal oocytes, which has a larger physical barrier that probably prevents it from participating

Table 1
The top five differentially abundant taxa that were identified using the ANCOM plugin in QIIME2. The W-value represents the number of ANCOM sub-hypotheses that have passed for each individual taxon. Note that the labellum vs. tarsi comparison only contained four total differentially abundant taxon. A full table is available in the supplementary files.

Comparison	Taxon	Group taxon is higher in	W-value
labellum vs. tarsi	*Chishuiella wautersiella*	labellum	1274
	Vagococcus	tarsi	1266
	Ignatzschineria	tarsi	1264
	Suttonella	tarsi	1202
labellum vs. oocyte	*Suttonella*	oocyte	746
	Tumebacillus	labellum	744
	Psychrobacter pulmonis	labellum	744
	Chishuiella wautersiella	labellum	725
	Pseudomonas	labellum	713
tarsi vs. oocyte	Enterobacterales	oocyte	935
	Psychrobacter pulmonis	tarsi	932
	Tumebacillus	tarsi	924
	Pseudomonas	tarsi	916
	Corynebacterium urealyticum	tarsi	899
April vs. February	*Providencia*	April	1093
	Ignatzschineria	February	1092
	Vagococcus	April	1091
	Wolbachia	April	1091
	Tumebacillus	April	1091
April vs. July	*Pseudomonas*	July	1163
	Wolbachia	April	1161
	Psychrobacter pulmonis	July	1160
	Actinomycetospora	July	1155
	Corynebacterium	July	1154
February vs. July	*Actinetobacter*	February	624
	Ignatzschineria	February	624
	Tumebacillus	July	624
	Providencia stuartii	July	620
	Comamonadaceae	July	619

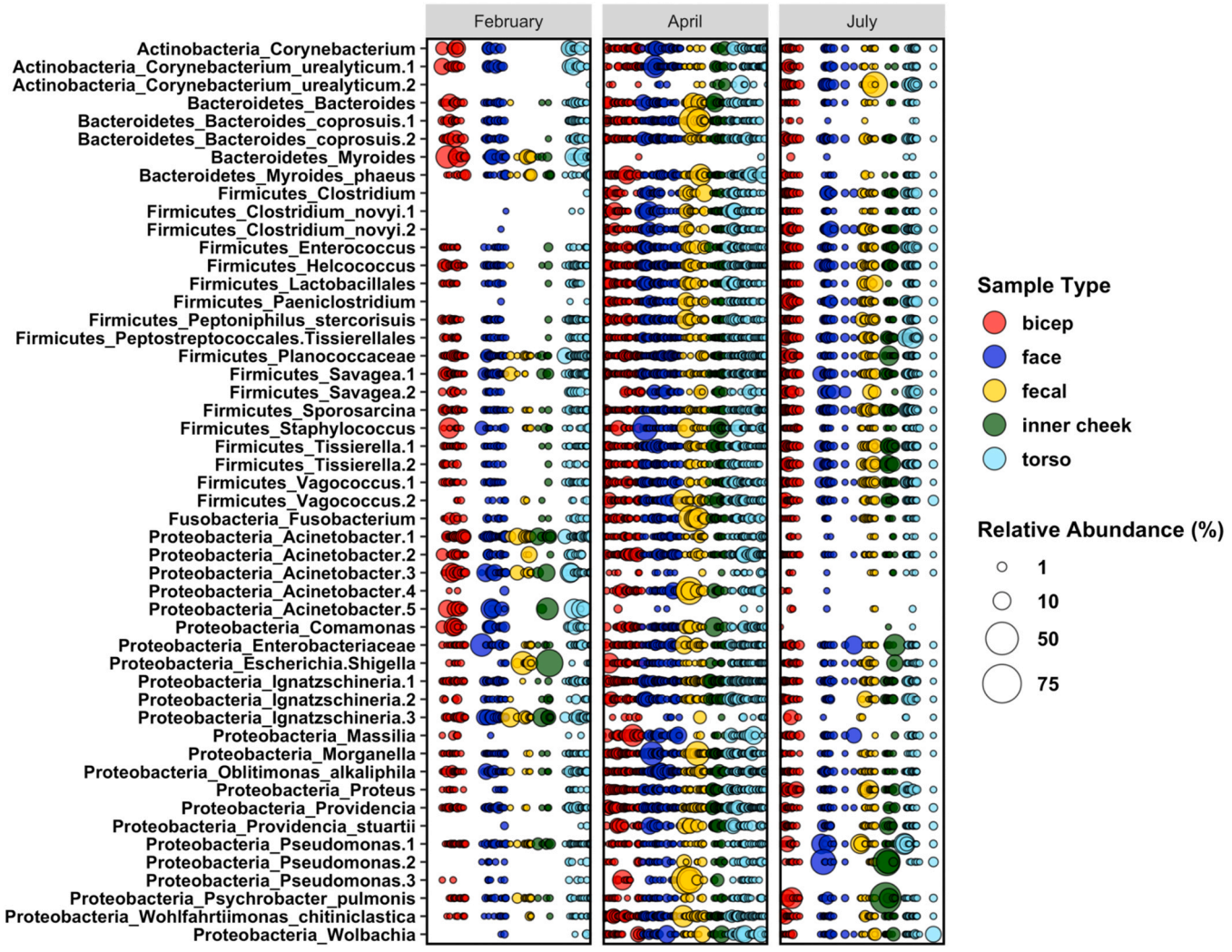

Fig. 4. A bubble chart of the relative abundance of the top 50 human-associated microbial taxa colored by sample type and separated by placement season. Taxa are sorted by phylum, and each taxon name contains the phylum and the taxonomically lowest identifiable name. Each column represents the microbial community of a single human sample within the indicated season, and each row shows the relative abundance of the specified taxon (with all bubbles in the row adding up to 100 %). Taxa with number assignments represent different amplicon sequence variants that were assigned to the same taxon.

in microbial transfer and the fact that bacteria present in the oocytes are there most likely due to transovarial transmission [83]. While contamination of oocytes during dissection is possible, steps were taken to minimize the possibility (flies were washed and sterile dissection techniques were employed, see methods).

3.5. How does the fly microbiome compare between seasons and species?

Beta diversity analyses show clustering by the season of fly collection, in which February is distinct from both April and July, the latter of which overlap (Fig. 3B). All seasons were significantly different from each other ($q = 0.001$ for all pairwise PERMANOVA comparisons for unweighted and weighted UniFrac metrics). The pseudo-F value (effect size) was higher for season than fly part for both unweighted (9.15 and 17.59 for part and season, respectively) and weighted (8.36 and 27.09 for organ and season, respectively) UniFrac metrics, indicating that season has a larger effect on the fly microbiome than the fly part. This seasonal effect may be due to less fly activity during cooler months [84], as the average April and July placement temperatures (20.21 °C and 27.71 °C, respectively) are closer together than they are to the average February placement temperature of 10.23 °C. Furthermore, it is possible that variation in species occurrence between seasons (Fig. 1A) may play a role in seasonal differences. Since there was also a statistical difference in the weighted and unweighted UniFrac metrics between fly species (Fig. S3), multivariate ADONIS tests [85] were used to understand whether season, species, or both were the predominant drivers of microbial community structure. Table S2 summarizes these results. In short, when only fly species or only season was considered, each of these variables were a significant driver of beta diversity for all tested metrics (Table S2). Results were still significant when testing for the interaction of these variables for all metrics except for weighted UniFrac. However, in every test, the R^2 value was higher for fly species than season. This indicates that while both variables significantly affect beta diversity, it is likely that fly species has a stronger influence on the fly microbiome than the season that they are collected in. To further investigate this, a more controlled study that manages the release of specific fly species within a climate-controlled decomposition environment would be required.

To better understand seasonal variation, pairwise analysis using the ANCOM plugin in QIIME2 identified a total of 135 differentially abundant features between flies collected in different seasons. Notable differentially abundant features include *Ignatzschineria*, which was more frequent in February, *Wolbachia*, which was more abundant in April, along with *Tumebacillus* and two features

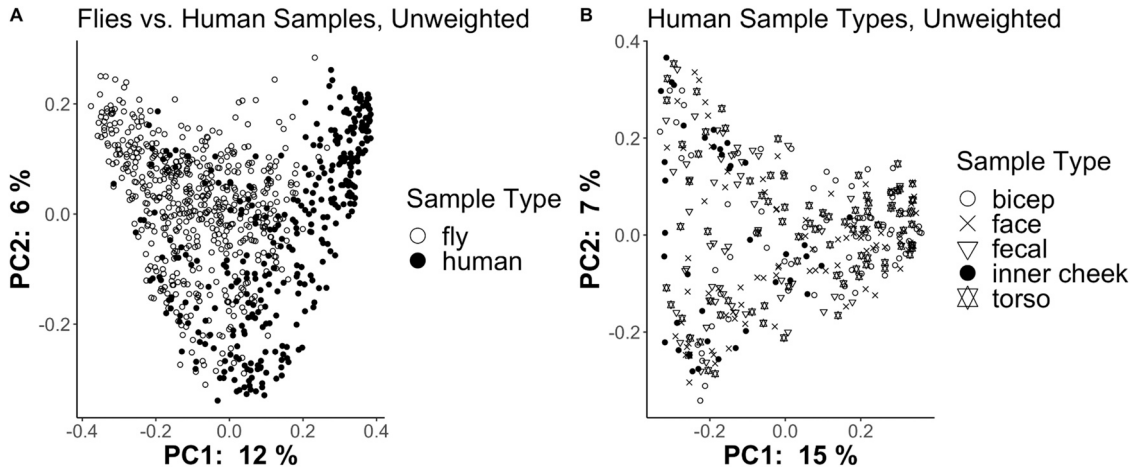

Fig. 5. Unweighted UniFrac beta diversity of human vs. fly samples (A) and the different sample types within the human data (B). PERMANOVA comparisons showed that the fly samples were significantly different from human samples ($p = 0.001$, 999 permutations, pseudo-F = 58.10), and pairwise PERMANOVA comparisons showed that some human sample types were significantly different from each other (fecal and inner cheek samples were different from each other as well as all other sample types, $q = 0.001$ for all unweighted comparisons, 999 permutations). Other human sample types (bicep, face, torso) were not significantly different from each other ($0.11 < q < 0.75$ for all comparisons, 999 permutations). The weighted UniFrac version of this can be seen in Fig. S5.

belonging to genus *Providencia*, which were more abundant in the warmer months April and July. While literature on the seasonal fly microbiome is lacking, one study by Wei et al. [76] did observe that the microbiome of *Lucilia sericata* differs between seasons, in which *Staphylococcus* increased in the spring, *Ignatzschineria* increased in the summer, and *Vagococcus*, *Dysgonomonas*, and an unclassified Acetobacteraceae increased in the fall. These results do not agree with those of our dataset (e.g., our results instead showed a differential increase of *Ignatzschineria* in February). There may be several reasons for this, including differences in geographic location, local animals and vegetation, solar irradiation, or that Wei et al. [76] did not conduct their study in a decomposition environment.

3.6. Do fly-associated bacteria appear in the human decomposition microbiome, and how does this differ between placement seasons?

To check whether the variable treatment of cadavers before placement (cooled, frozen, or both) affected the microbial community structure, beta diversity analyses shown in Fig. S1 were performed. Although it didn't appear that there was a significant visual trend, pairwise PERMANOVA results were $q < 0.05$ for all comparisons. In studies involving human subjects, researchers are unfortunately unable to control for some variations due to lack of donors. Because of this, the authors of this study were unable to control cadaver treatment before placement. However, the focus of this manuscript was to examine the influence of the fly microbiome on human cadaver microbial community assembly throughout different seasons. Since there was a mix of cooled, frozen, and cadavers that underwent both treatments within the seasons, the authors do not believe that this variable treatment has significantly affected analyses. Taxonomic analysis showed that many of the same bacteria phyla found in the fly data were also found in the human sample types, including Actinobacteria, Bacteroidetes, Firmicutes, and Proteobacteria (Fig. 4). An exception is the presence of one ASV classified as taxon *Fusobacterium* within phylum Fusobacteria. This is unsurprising, as *Fusobacterium* is typically a human pathogen [86]. At a lower taxonomic level, many of the fly genera were similarly found in the human samples, including common fly associated bacteria like *Wolbachia*, *Ignatzschineria*, and *Wohlfahrtiimonas* (Fig. 4). Beta diversity analyses using the unweighted UniFrac metric showed that fly sample types clustered away from human sample types (Fig. 5A, PERMANOVA $p = 0.001$ for both unweighted and weighted UniFrac). Fecal and inner cheek sample types were significantly different from each other and from all other sample types for both unweighted UniFrac and weighted UniFrac for all pairwise PERMANOVA comparisons ($q = 0.001$, 999 permutations) except for one case, in which the fecal and inner cheek samples were not significantly different when compared using the weighted UniFrac metric ($q = 0.126$). In all comparisons for both metrics, the bicep, face, and torso samples were not significantly different from each other ($0.11 < q < 0.75$ for all comparisons, 999 permutations). This indicates that the area of skin in which flies interact with may not affect the microbes they acquire, but that their interaction with other more distinct sample types such as feces and the inner mouth may have greater influence. Since flies are more likely to oviposit in moist, protected areas like the mouth, ears, nose, and anus [87], it is worthwhile to consider the structure of these microbial communities when understanding the dynamics of microbial transfer between flies and human remains. Because of the many fly-associated genera found within the human decomposer microbiome, SourceTracker2 was used to track the transfer of fly microbes more accurately onto humans during decomposition. The purpose of this was to track a one-way movement of microbes from designated "sources" (labellum, tarsi, and oocyte) to "sinks" (bicep, face, fecal, inner cheek, and torso) [57]. Source tracking analyses showed that in February, the tarsi microbiome is a higher contributor to the human decomposition microbiome, with the labellum microbiome acting as a smaller source and the oocyte microbiome a relatively nonexistent source (Fig. 6A). As the months become warmer, the labellum source proportion increases, the tarsi source proportion decreases, and the oocyte microbiome begins to contribute to the human decomposition microbiome, albeit minimally (Figs. 6B and 6C). Therefore, flies are likely a source of microbes during human decomposition, and the fly source part (labellum, tarsi or oocytes) varies between seasons. This study suggests that the fly-borne bacteria *Ignatzschineria* may be spread by the fly to the cadaver through the tarsi. In general, these seasonal trends held regardless of the human sample type, with one notable exception being that tarsi appeared to contribute a higher source proportion of the inner cheek community in July (Fig. 6C). These trends may be explained by the variable numbers of flies swarming the cadavers between seasons. In the colder months (represented by the February placements) typically there is less fly activity (abundance and diversity). In these cases, sometimes there is no fly activity until temperatures are warmer, which often does not occur until the peak daily temperature is reached the day after the cadaver was placed. During the warmer months (represented by

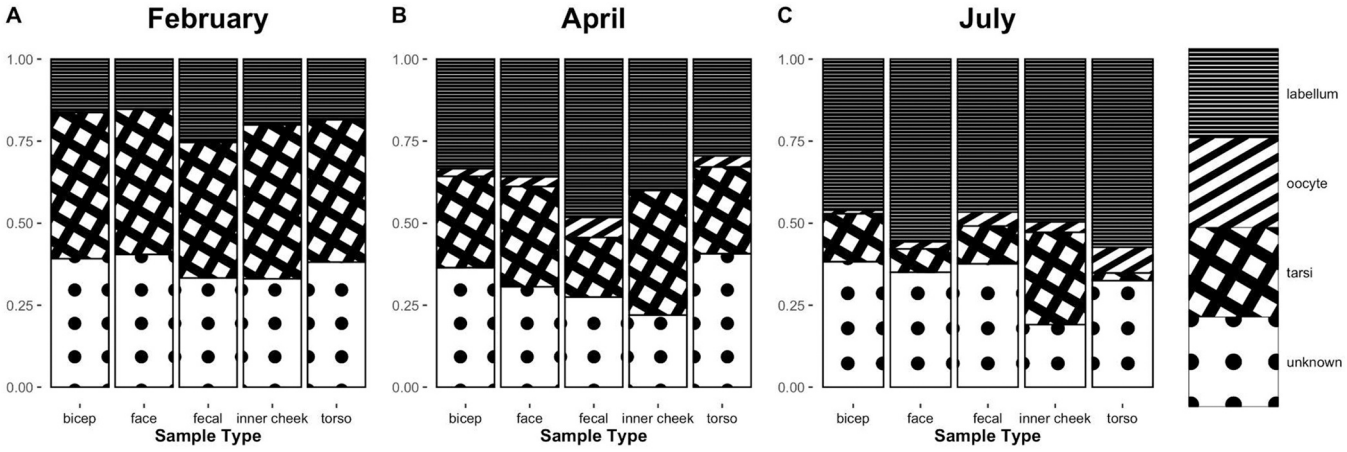

Fig. 6. The predicted source proportions of fly organ microbes on human sample types, separated by placement season.

the April and July placements), up to hundreds of flies can swarm the cadavers within minutes of placement. The higher temperature results in an increased amount of interaction between flies and humans, which may explain the differences in source proportions between the tested months. Additionally, temperature can also affect insect activities such as feeding with insect appetite potentially decreased during cooler periods. Flies that are not feeding would not lower their proboscis and would not regurgitate as much as flies that are feeding.

4. Conclusions

The fly microbiome observed in our dataset had several commonalities with other fly microbiome studies, indicating a "universal" fly microbiome that persists even in a decomposition environment. Despite this, there were still notable differences between fly parts and seasons. While there was a statistically significant signal between the labellum, tarsi, and oocyte, seasonal placement had a stronger effect on the fly bacterial communities. The authors recognize that fly species occurrence may play a role in seasonal differences. Testing this would require an experiment that controls for the release of specific fly species during each season. Furthermore, flies act as substantial bacterial sources of the human decomposer bacterial community, with the source contribution per fly part varying based on the time of year. This study has characterized the fly microbiome by organ during different seasons, and it has provided evidence that a transfer of microbes from flies to humans during decomposition influences the human cadaver microbial community assembly.

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CRediT authorship contribution statement

Sibyl R. Bucheli, Keli King, and Cameron Huhn contributed to study conception, design, and data collection. Sophia Montoya and Alexandra Emmons contributed to preliminary data analyses. Heather L. Deel, Sibyl R. Bucheli, Aaron M. Lynne, and Jessica L. Metcalf contributed to final analysis planning, conducting analyses, data interpretation, and manuscript writing and editing. All authors contributed to the editing and review of the manuscript. All authors have read and approved of the final manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data Availability

All data, analyses code, and supplemental files are provided in https://github.com/Metcalf-Lab/fly_human_2021_Deel.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.forsciint.2022.111425.

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