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Article

Microbiomes of Two Pest Fly Species of Pennsylvania Mushroom Houses

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Simple Summary: Flies inhabit mushrooms or consume them incidentally through their lifecycle. In wild conditions, these flies are part of the recycling process and are not considered pests. However, in commercial mushroom houses, these flies can become problematic, as compost pests, physical damage of the mushroom production through consumption, or indirectly as vectors of pathogens or nematodes. Here we describe the bacterial associates of two fly pest species of Pennsylvania mushroom houses.

Abstract: Mushroom cultivation vastly improves the yield of mushrooms under optimized, controlled conditions, but may be susceptible to opportunistic colonization by pest species that can establish themselves, as well as the pathogens and pests they may transmit. Here we describe our investigation into the bacterial communities of adult *Lycoriella ingenua* (Diptera: Sciaridae) and *Megaselia halterata* (Diptera: Phoridae) collected from button mushroom (*Agaricus bisporus)* production houses in Pennsylvania. We collected adult flies and sequenced the hypervariable v4 region of the bacterial 16S rRNA using the Illumina MiSeq. The most abundant bacterial genus detected in both species was *Wolbachia*, but phylogenetic analysis revealed that the infections are from different clades. Future studies include the characterization of *Wolbachia* infections on fly behavior and biology, comparison of microbial diversity of fly species colonizing wild mushrooms, and other microbiota that may contribute to the success of certain pest fly species.

Keywords: Mushroom fly; microbiome; *Lycoriella ingenua*; *Megaselia halterata; Wolbachia*

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1. Introduction

Fungi are integral components of many ecosystems. Many fungi produce sporocarps, macroscopic structures in which sexual spores develop and from which spores are released. Fungi play many roles in nature: predators, parasites, mutualists, and/or recyclers. A given fungal species may be a mycorrhizal companion of nearby plants, food and shelter for developing invertebrates, and may itself be parasitized by microorganisms, while also consuming bacteria and benefiting from bacterial breakdown products. Mycetophagous flies in turn may consume fungal material (mycelia or fruiting bodies) and utilize volatiles from fungal pathogens or bacterial breakdown products to select optimal oviposition sites [1].

Wild sporocarp-forming fungi are populated with a rich diversity of dipterans [2]. In the northeastern United States the fruiting structures of the fungal genus *Agaricus* are predominantly colonized by drosophilids, and to a lesser extent, by phorids and tipulids [3]. In contrast, only a handful of fly species are considered economic pests of mushroom production worldwide. Fly pests are predominantly from the families Phoridae, Sciaridae, and Cecidae (in some places Drosophilidae are also problematic) [4,5]. In Pennsylvania mushroom farms, the two major pest species are *Lycoriella ingenua* (Dufour 1839) and *Megaselia halterata* (Santos Abreu, 1921) [6].

Agricultural monocultures are optimized for maximum crop yield, but are also susceptible to opportunistic colonization by pest species that can establish themselves, as well as the pathogens and

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pests they may transmit. Mushroom houses serve as an ideal experimental environment in which to study the dynamics of microbe-fly-cultivated crop interactions when conditions are optimized for mushroom crop production. Here we describe our investigation into the bacterial communities of adult *Lycoriella ingenua* (Diptera: Sciaridae) and phorid *Megaselia halterata* (Diptera: Phoridae) collected from button mushroom (*Agaricus bisporus)* production houses in Pennsylvania.

These two pest species have distinct but overlapping biologies. *L. ingenua* consume the compost material (and any associated microbes) prior to addition of mushroom spawn [7]. Once the compost is fully colonized by *Agaricus bisporus*, the populations of *I. ingenua* decline [8]. In contrast, populations of *M. halterata* thrive on mycelial growth, gradually building up from Spring until Fall and then declining when mushrooms are harvested and beds replaced [9]. We predicted that there would be some overlap in bacterial community composition between the two fly species, but we suspected there would be differences that might be unique to each fly species. We collected specimens of each species, extracted their nucleic acids, and sequenced the bacterial 16S rRNA gene sequences using the Illumina MiSeq sequencing platform. While we did detect some overlap in bacterial diversity, we additionally identified two phylogenetically distinct *Wolbachia* sequences.

2. Materials and Methods

2.1. Collection Sites and Sampling

We collected *Lycoriella ingenua* and *Megaselia halterata* adults by aspiration from two mushroom production houses in Kennett Square, Chester County, PA, at two times (May and October of 2014; Table 1). Flies were collected in separate vials by species (*L. ingenua* or *M. halterata*) and transported alive on ice to University Park, PA to prevent damage to the nucleic acids. Flies were then placed at -80 C until processed.

Table 1. Bacterial metagenomic sample table. SampleID, Species, collection date, number of flies sequenced, individually or in pools, and sequencing targets are listed.

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SampleID	Fly species	Collection date	Individuals (N)	Sequencing Target
E05Sc051614Li01 E06Sc051614Li02 E07Sc051614Li03 E08Sc051614Li04 E09Sc051614Li05 E10Sc051614Li06 E11Sc051614Li07 E12Sc051614Li08 F01Sc051614Li09 F02Sc051614Li10	*L. ingenua*	05/16/2014	Individuals (10)	Bacterial 16S rRNA
F03Sc091814Li01 F04Sc091814Li02 F05Sc091814Li03 F06Sc091814Li04 F07Sc091814Li05 F08Sc091814Li06 F09Sc091814Li07 F10Sc091814Li08 F11Sc091814Li09 F12Sc091814Li10	*L. ingenua*	09/18/2014	Individuals (10)	Bacterial 16S rRNA
C09Ph051614Mh01 C10Ph051614Mh02 C11Ph051614Mh03 C12Ph051614Mh04 D01Ph051614Mh05 D02Ph051614Mh06 D03Ph051614Mh07	*M. halterata*	05/16/2014	Individuals(7)	Bacterial 16S rRNA
D04Ph091814Mh04 D05Ph091814Mh05 D06Ph091814Mh06 D07Ph091814Mh11 D08Ph091814Mh12 D09Ph091814Mh13 D10Ph091814Mh14 D11Ph091814Mh15 D12Ph091814Mh16 E01Ph091814Mh17 E02Ph091814Mh18 E03Ph091814Mh19 E04Ph091814Mh20	*M. halterata*	09/18/2014	Individuals(13)	Bacterial 16S rRNA

2.2. Sample Processing for Bacterial 16S rRNA Sequencing

We extracted genomic DNA from 40 individual flies (Table 1) and sequenced the v4 hypervariable region of the bacterial 16S ribosomal RNA gene to describe the bacterial community composition. DNA extractions were conducted using the Omega E.Z.N.A. Tissue DNA kit (SKU\#: D3396) following the manufacturer's suggested protocol. Barcoded PCR of the v4 region of the bacterial 16S rRNA gene, followed by sequencing on the Illumina MiSeq platform.

2.3. Analysis of Bacterial Communities of Flies

Sequences were aligned, filtered to remove chimeric sequences, and analyzed in RStudio using Dada2 and Phyloseq, followed by further refinements using the R/Bioconductor framework "miaverse" [10]. Reads were assigned taxonomic identity using the Dada2 taxonomy assigner and Silva (v138) reference database of eubacterial 16S ribosomal RNA sequences [11–13]. After an initial analysis, we detected taxa that matched mitochondrial sequences or were suspected to be contaminants from other samples sequenced in the same run (see "Post-Illumina sequence confirmation and phylogenetic placement of *Wolbachia* sequences"). Thereafter, we filtered out reads matching "mitochondria", "*Rickettsia*", and "*Rickettsiella*" before continuing with diversity analyses.

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Shannon and inverse Simpson indices were used for measuring richness and evenness. Statistical comparisons within species were performed using the Kruskal-Wallis test. Community dissimilarity (Bray-Curtis index) was evaluated between groups. Multidimensional scaling analyses (MDS) were calculated and plotted to visualize bacterial community structure between groups using Phyloseq. Statistical comparison between groups was performed to run permutational multivariate analyses of variance (PERMANOVA using 999 permutations). The significance level was set to 0.05.

2.4. Post-Illumina Sequence Confirmation & Phylogenetic Placement of Wolbachia Sequences

Our samples were sequenced with samples from other arthropod studies (two different mosquito species and a tick species). Because of this, it was important to confirm the presence of the three bacterial genera that were also detected in one or more of those arthropod hosts. We used PCR primers for *Rickettsia*, *Rickettsiella*, and *Wolbachia* [14,15]. We did not detect the presence of *Rickettsia* or *Rickettsiella*, both of which were taxa in high abundance in tick samples sequenced on the same run. However, we did detect fragments of *Wolbachia* 16S rRNA using primers W-Specf (5'\-CATACCTATTCGAAGGGATAG\-3') and W-Specr (5'\-AGCTTCGAGTGAAACCAATTC\-3') to amplify a 438 bp fragment [14]. Amplicons from four fly samples (2 of each fly species) were gel-separated, purified, and submitted for Sanger sequencing. Amplicons were aligned to known GenBank deposited sequences of *Wolbachia*, trimmed to 330 bp to eliminate indels, and phylogenies estimated using Maximum Likelihood with MEGA X using the best-estimated model of evolution selected by jmodeltest [16,17].

3. Results

3.1. Bacterial Community Composition

In total, 2,389,953 16S rRNA reads passed quality control and chimera checking. Read counts were higher from *L. ingenua* (1,695,053) than *M. halterata* (694,900) (Figure 1). Reads matching *Wolbachia* were found in all individual flies from both species. *Wolbachia* was the dominant taxon in all individuals of *L. ingenua*. In *M. halterata* the abundance of *Wolbachia* was variable and did not always represent the dominant taxon. *Klebsiella*, *Pseudomonas*, *Ralstonia* were often more abundant in *M. halterata* than in *L. ingenua*) (Figures 2 and 3).

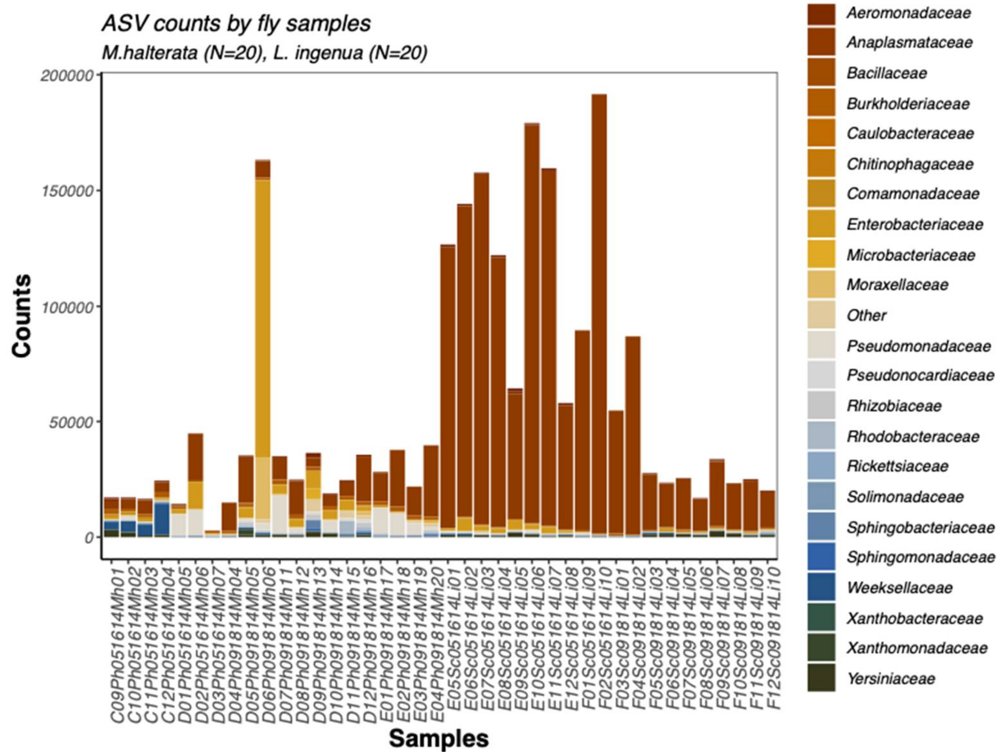

Figure 1. Absolute read counts by fly sample sequenced on Illumina Mi-Seq.. Samples of *Megaselia halterata* (Phoridae) = C09-E04. Samples of *Lycoriella ingenua* (Sciaridae) = E05-F12.

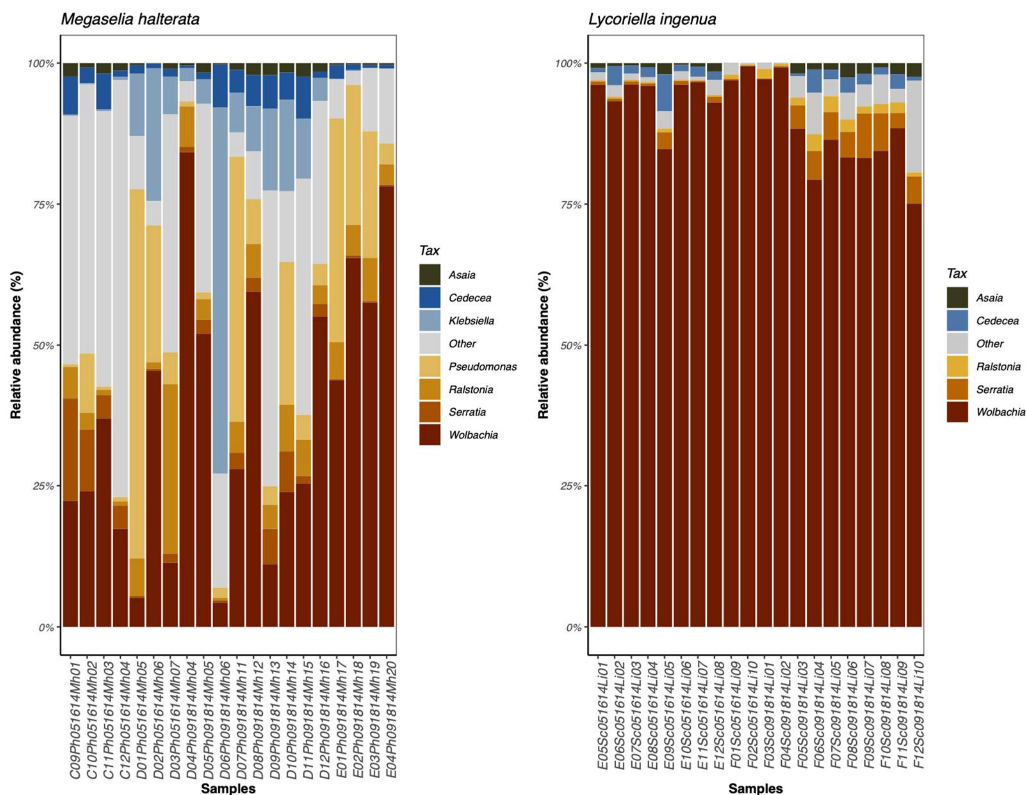

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Figure 2. Relative read abundance of bacterial genera by fly species. Relative bacterial read abundance by individuals for each species of fly (Samples of *Megaselia halterata* (Phoridae) = C09-E04. Samples of *Lycoriella ingenua* (Sciaridae) = E05-F12).

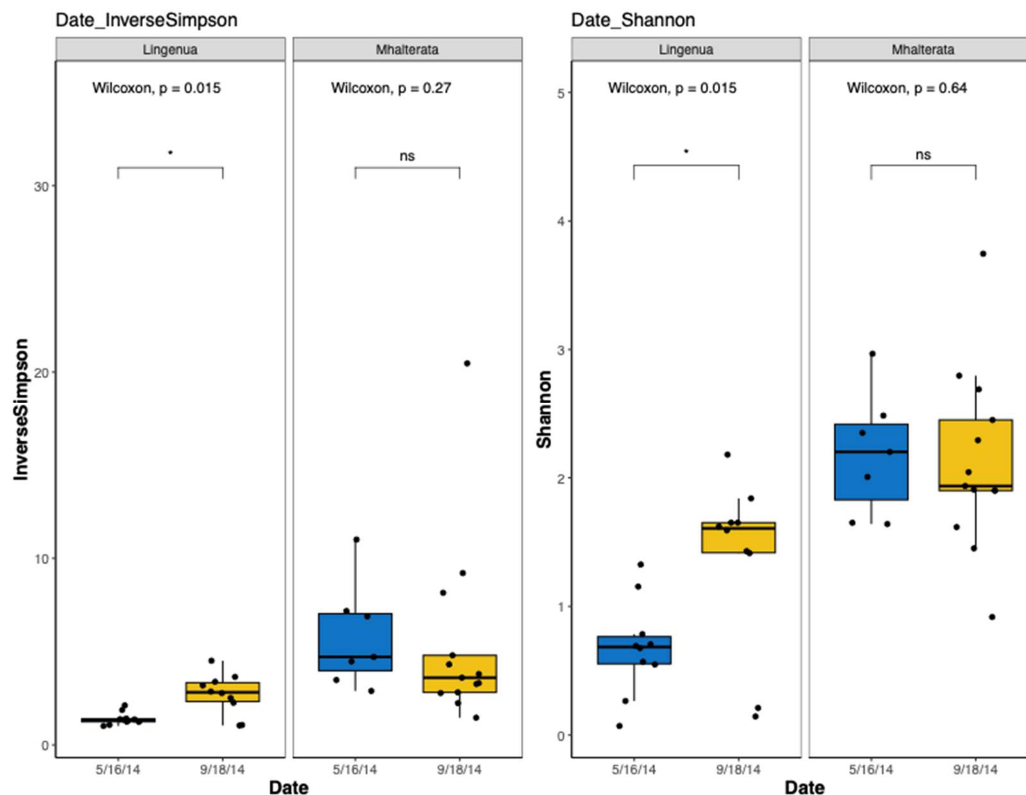

Figure 3. Comparison of alpha diversity by date within each fly species using Inverse Simpson and Shannon indices. There was a significant difference in diversity in *L. ingenua* samples collected in May versus September. This difference in diversity was not observed in *M. halterata* between collection dates.

3.2. Bacterial Diversity

L. ingenua microbiota are not evenly distributed and fewer taxa (lower richness) were identified than observed in *M. halterata*. We detected significant differences in diversity between collection times in *L. ingenua*, but not in *M. halterata* (Figure 4). While the alpha diversity measures of *L. ingenua* individuals differed between May and September collections, this was not the case for *M. halterata* (most *M. halterata* bacterial taxa clustered together regardless of dates).

When we examined the beta diversity, we observed that the diversity measures of the two species were distinct from each other, although there was some clustering between fly species that corresponded to the fall collection (Figure 5). We confirmed that there was a significant interaction between fly species and collection date using a Permanova analysis (Species p=0.001; Date p= 0.022; Species*Date p=0.008). We therefore analyzed the two species separately to confirm the effect of the collection date. In both species, there was a significant effect of collection date (Li, R2=0.36726, p>0.005; Mh, R2=0.13511, p>0.003).

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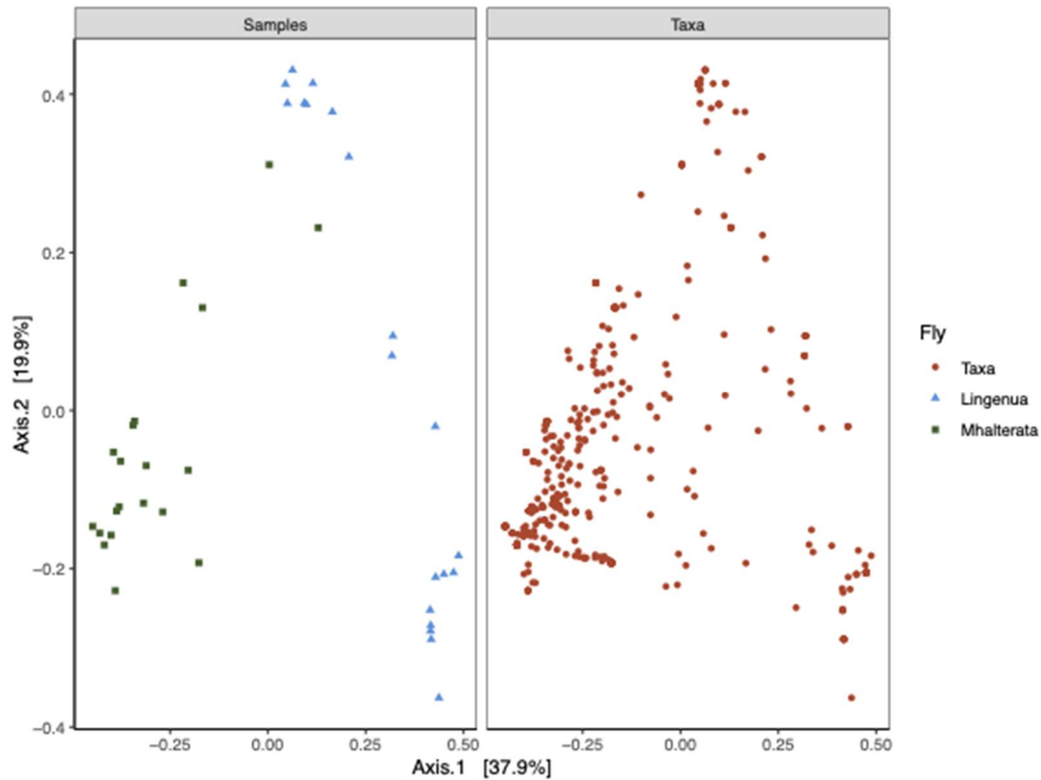

Figure 4. Split plot of Multidimensional Scaling (MDS) with a Bray Curtis dissimilarity measure of fly species and bacterial taxa. Fly samples are plotted on the left while bacterial taxa are plotted on the right.

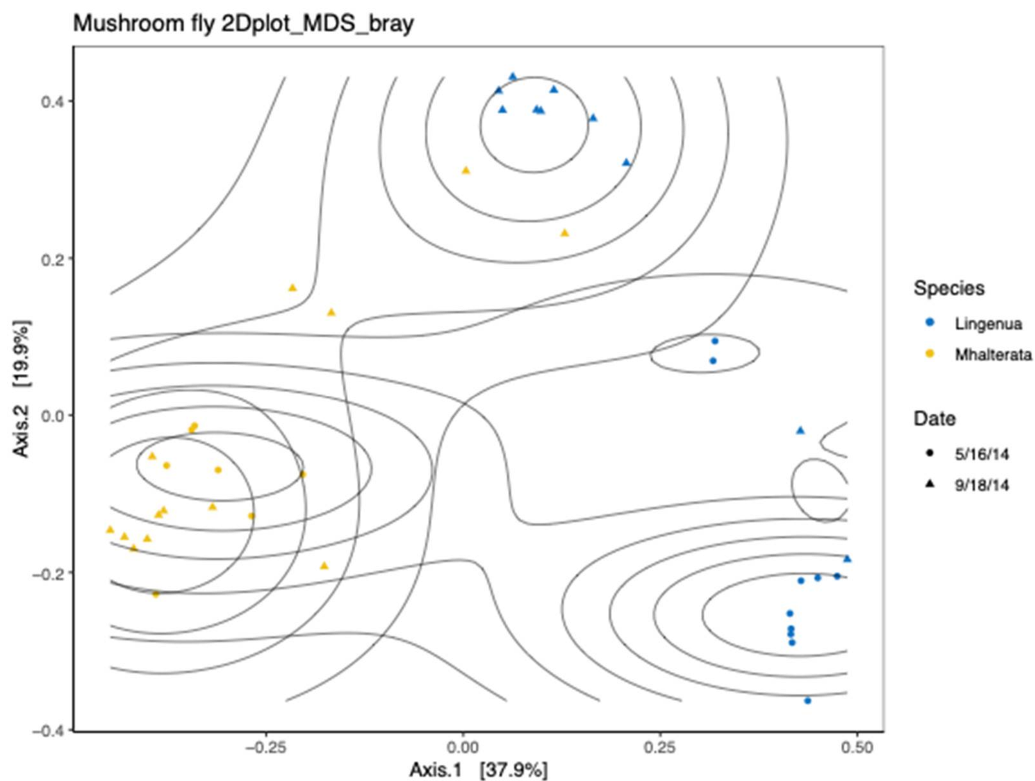

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Figure 5. Two-dimensional density plot for both fly species. Data are plotted using a Multidimensional Scaling Analysis (MDS) with a Bray Curtis dissimilarity measure. Samples closer together are more similar in diversity than those that are farther away.

3.3. Sequence Confirmation and Phylogenetic Placement of Wolbachia Sequences

Fragments of *Wolbachia* 16S rRNA sequences from four samples (two from each fly species) were amplified, gel-purified, and submitted for Sanger sequencing. We confirmed that the *Wolbachia* sequences identified in the dataset were not due to sequence contamination and that the isolates from each fly species were from phylogenetically distinct clades (Supergroup E for *L. ingenua* and Supergroup B for *M. halterata*) (Figure 6).

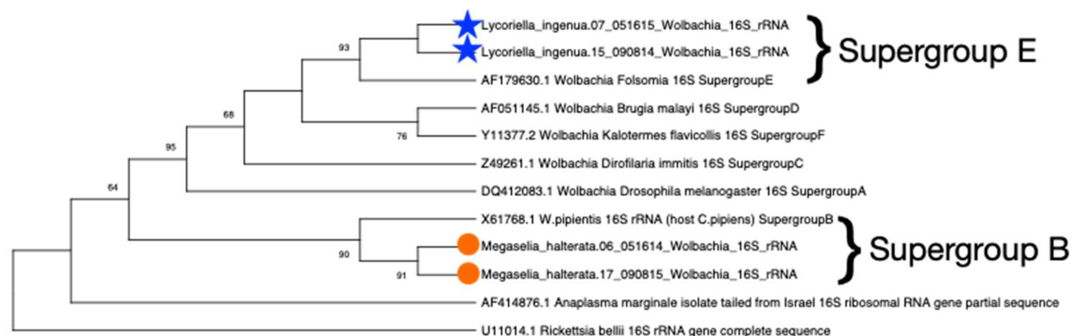

Figure 6. Maximum likelihood phylogenetic tree of *Wolbachia* 16SrRNA sequences from each fly species. Analyses were conducted using MEGA X. The evolutionary history was inferred using the Maximum Likelihood method and the Tamura-Nei model. The bootstrap consensus tree was inferred from 1000 replicates. Branches with less than 50% bootstrap support are collapsed. Initial trees are obtained by Neighbor-joining and BioNJ algorithms. Evolutionary rate differences among sites were modeled using gamma distribution with the inclusion of some evolutionary invariable sites (+G, +I). All positions with less than 95% coverage were excluded. The trimmed and aligned fragment length was 330 bp. *Anaplasma marginale* and *Rickettsia bellii* were outgroups. Lm07 and Lm15 (stars)= *Lycoriella ingenua*; Mh06 and Mh17 (circles) = *Megaselia halterata*. Supergroups E and B represent *Wolbachia* Supergroups into which the fly *Wolbachia* sequences clustered.

4. Discussion

Bacterial community compositions were distinct for each fly pest species. We also observed an effect of collection date, particularly in *L. ingenua*. The two collection dates were chosen to compare spring versus fall population increases and fall mating behavior may have contributed to the difference in bacterial communities. It has been speculated that *M. halterata* adults, which leave the mushroom houses in fall to mate, may maintain populations outside of mushroom houses during the fall, although evidence of phorid presence was not detected in adjacent residential properties [18].

Because bacterial read counts were much higher from *L. ingenua* than *M. halterata* (Figure 1), it was important to consider this when interpreting differences in microbial communities between fly species. For instance, *Serratia* was found in higher absolute abundance in *L. ingenua,* but this only accounted for proportionally less than 10% of the total OTU abundance. On the other hand, the dominant bacterial taxon detected in both fly species was confirmed to be *Wolbachia.* Absolute and relative abundance of *Klebsiella, Pseudomonas*, and *Ralstonia* were higher in *M. halterata* than *L. ingenua.*

Wolbachia occurred at higher relative (and absolute) abundance in *L. ingenua* than in *M. halterata*. It is not unusual to find *Wolbachia* in fly species. However, phylogenetic analysis suggests that the *Wolbachia* found in both fly species may have been acquired independently. *Wolbachia* sequences contained in the fly species were determined to be from different clades. The presence of *Wolbachia*

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in both fly species was confirmed (post-Illumina sequencing) because of a concern that the sequences represented contamination from mosquito samples that were also sequenced in the same run. However, while the *M. halterata Wolbachia* was from a similar clade to *Culex pipiens Wolbachia*, it was distinct from the *Wolbachia* sequenced from the mosquito samples. Further, the *Wolbachia* identified in *L. ingenua* was from a completely different cluster (within the Supergroup E clade), a clade that has been previously associated primarily with springtails and several mite species [19,20]. One predatory mite (*Hypoaspis aculeifer*) known to be an effective biocontrol agent against both species of flies, was not observed or known to be in these mushroom houses, but even if it was present, it is not a species known to harbor *Wolbachia* [21,22]. Since the sequencing was conducted on whole flies (flies were too small to dissect for sufficient DNA for sequencing), we cannot exclude the possibility that the *Wolbachia* detected came from infected springtails or mites that may have been consumed by fly larvae in the mushroom mats. Whether or not the *Wolbachia* found in *L. ingenua* existed in the flies as a co-evolved associate, or acquired through horizontal transfer through interactions with other organisms in mushroom beds (e.g. springtails or mites) needs further research.

Lastly, *Pseudomonas* is a ubiquitous bacterial taxon and several species of *Pseudomonas* have been described from mushroom farms. Its presence was therefore not a surprise in our sequence data. While some species of *Pseudomonas* are important enhancers of mushroom development (metabolizing compost compounds that might otherwise inhibit *A. bisporus* primordial development), other species (e.g. *P. tolaasii* and *P. reactans*) are known pathogens [23]. Although we detected *Pseudomonas* in both fly species, we did not isolate or characterize them and cannot ascribe their nature as pathogenic, beneficial, or commensal within the mushroom house microbiome.

4.2. Limitations of the Study

Because bacterial metagenomic sequencing was performed on samples collected in different years, we cannot make any conclusions about the interactions of the bacteria in the microbial communities of their respective fly host. We cannot speculate whether the *Wolbachia* detected in this study caused sex ratio distortion or reproductive effects because we did not separate the males or females. We do not know the extent to which the microbial communities of flies and the mushrooms are shared, nor how they influence the behavior of the flies, nor can we determine the interactions of fly microbiota on secondary ecological interactions such as on parasitoids of mushroom flies, springtails, predatory mites, or nematodes.

4.3. Future Studies

Cultivated button mushroom farming represents a rich microbial ecosystem under fairly homogeneous environmental conditions. The current study connects one more piece of the multi-trophic ecological puzzle, but many questions are still unanswered. For instance, can the interactions and dynamics of mushroom flies with other microbial (e.g. viruses or nematodes) or invertebrate associates be used to facilitate the biocontrol of flies, or bacterial pathogens, or fungal pathogens of mushroom houses?

The purpose of the exploration of mushroom fly microbial dynamics was to identify biocontrol options in a cultivated setting. A broader ecological question we could not ignore was: What are the factors that dictate which fly species becomes a pest? Wild mycophagous flies are dependent on a resource whose abundance is heavily affected by rainfall and other variables. Thus, resource unpredictability would likely favor polyphagy, not host specialization in mycophagous flies [1]. The diversity of mycophagous fly taxa encountered in wild mushrooms reflects this and represents an arena for resource competition. Wild mushrooms (*Agaricus* spp. in particular) in the Northeastern United States are largely colonized by mycophagous drosophilids (*Drosophila* and *Leucophenga* spp.), wood gnats, mushroom flies, and crane flies [1,3]. While a limited food source (e.g. single basidiocarp) might result in inter- and intraspecies competition and subsequent reduction in size, an effectively inexhaustible food source (such as a mushroom house) would likely favor mushroom flies.

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In theory, any mycophagous fly in the vicinity should benefit from such an abundance of resources. Yet, in Pennsylvania mushroom houses only Phoridae and Sciaridae (specifically the two species in this study) are of concern as pests, although in other regions of the country, other fly species may become problematic (Cecidomyiidae; *Mycophila speyeri)* and *Heteropeza pygmaea*), house and stable flies (as nuisance pests of compost heaps), and members of the Drosophilidae) [5]. Some cultivation practices and control practices may exclude some of these species, but one future study includes an in-depth investigation of the multitrophic dynamics of fly colonization of wild mushrooms in adjacent wooded areas to identify possible explanations for the exclusion/establishment of other fly species in cultivated settings.

Mushroom cultivation began in the 1600s, but structures or caves were not used until the early 1900s [24]. Modern mushroom houses were started in the early 1900s, but were quickly plagued by sciarid pests [25]. While earlier attempts at control included chemical applications, the quick development of resistance necessitated changes in cultivation practices and biocontrol agents as integrated pest management strategies [26,27]. Cultivation practices such as compost pasteurization and the use of chemicals and biocontrol agents (e.g. predatory mites, entomopathogenic nematodes and fungi) can help control fly pests, but exclusion is preferred [21,28,29]. It should be noted that pasteurization alone may not be sufficient, as adult female sciarid (*L. ingenua*) are attracted to compost and the volatiles released by pathogenic molds [30].

What role *Wolbachia* play in the lifecycle of either of these fly species is unknown. Further studies would include attempts to cure colonized flies of *Wolbachia* infections and determine whether/how this affects biology, behavior, or pathogen vector competence. We can also determine the population genetics of the *Wolbachia* isolates in mushroom houses and in wild mushroom populations.

Author Contributions: Conceptualization and sample collection, JS and JR; methodology, and literature review, JS and IS; data analysis and visualization, JS; manuscript writing and editing, JS, IS, JR; funding acquisition, JS and JR. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: Sanger sequences of *Wolbachia* 16S rRNA fragments can be found at Genbank Accession # PP549140-PP549143. Illumina sequences of bacterial 16S rRNA sequences from fly samples are at Genbank under Bioproject Accession # PRJNA1093092.

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Conflicts of Interest: The authors declare no conflicts of interest.

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