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Comparing Approaches for Capturing Bacterial Assemblages Associated with Symptomatic (Bacterial Blotch) and Asymptomatic Mushroom (*Agaricus bisporus*) Caps

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ABSTRACT

Bacterial blotch is a complex of diseases, caused by *Pseudomonas tolaasii* and other *Pseudomonas* spp., whose growth may be influenced by other microorganisms associated with mushroom caps. The objectives of this work were to (i) compare methods for extracting DNA from mushroom caps to assess bacterial composition, and (ii) describe and quantify the composition of bacteria in and on symptomatic and asymptomatic mushroom caps. Symptomatic and asymptomatic mushrooms were collected at an organic farm in Pennsylvania and processed. Bacteria were removed from mushrooms by sonication to target surface-associated bacteria (washate), from whole mushrooms postsonication to target bacteria within or tightly adhering to the mushrooms (post-washate), or from whole macerated mushrooms to target all mushroom-associated bacteria in one sample (whole). Bacterial composition was determined through sequencing the V4 hypervariable region of the 16S rRNA gene on an Illumina MiSeq. Bacterial composition differed significantly based on mushroom health status and

community removal method. Regardless of sampling approach, *Proteobacteria* and *Bacteroidetes* appeared as the most dominant phyla. In comparing the three extraction methods, higher Shannon diversity and Pielou's evenness were observed for the washate and post-washate methods compared with whole mushroom sampling. Additionally, higher Shannon diversity, operational taxonomic unit richness, and Pielou's evenness were found for the asymptomatic mushrooms when compared with symptomatic mushrooms. *Pseudomonas* spp. were overrepresented on mushroom surfaces (washate method), regardless of health status. This research made strides in describing the mushroom cap microbiome and established sampling methods that will allow researchers to target studies and isolation of specific bacterial taxa.

Keywords: 16S rRNA gene, *Agaricus bisporus*, bacteriology, biological control, blotch disease, microbiome, plant pathology, *Pseudomonas*

Agaricus bisporus, known as the white button mushroom, is the most cultivated mushroom in the Americas, Australasia, and parts of Europe (Chang and Wasser 2017; Royse et al. 2017). Interactions with bacteria are an essential feature of *A. bisporus* cultivation and

can impact mushroom development and health, particularly when those bacteria are pathogenic (Kertesz and Thai 2018). Bacterial blotch of *A. bisporus*, a complex of diseases that results in a rapid browning or blotching and deterioration of mushroom caps, is one of the most economically important diseases or disease complexes in *A. bisporus* production (Navarro et al. 2018; Aslani et al. 2018). These diseases are caused by at least 14 species of fluorescent *Pseudomonas* (members of the phylum *Proteobacteria*), including *P. tolaasii*, which has been studied for over 100 years (S. Martins et al., unpublished data) (Paine 1919; Tolaas 1915). The disease(s) can develop rapidly, in part, because mushroom tissue provides an ideal environment for bacterial growth and proliferation, because tissue moisture content is high (water activity values typically ≥ 0.98) and tissue pH is often near neutral (Martinez-Carrera et al. 1998; Rossouw and Korsten 2017; Venturini et al. 2011). Inoculation of pathogenic bacterial strains on *A. bisporus* caps can lead to rapid disease development within approximately 15 h (S. Martins et al., unpublished data). Recently, Navarro et al.

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(2018) estimated reduced yields in Spanish crops of *A. bisporus* up to 15% caused by species of *Pseudomonas*, including *P. tolaasii*. Several previous reports targeted the isolation and molecular identification of *Pseudomonas* species (Godfrey et al. 2001; Munsch et al. 2002; Navarro et al. 2018; Siyoum et al. 2016; Tolaas 1915) (S. Martins et al. *unpublished data*). These studies applied culture-dependent methods to identify *Pseudomonas* spp. associated with blotch disease in *A. bisporus*.

In contrast, some *Pseudomonas* spp. are beneficial to *A. bisporus* growth. For instance, fluorescent *P. putida*, commonly found in compost and casing layers, is critical for initiating *A. bisporus* fruiting bodies (Noble et al. 2003; Zarenejad et al. 2012). Additionally, many other *Pseudomonas* spp., including *P. putida*, *P. fluorescens*, *P. brassicacearum*, *P. chlororaphis*, *P. koreensis*, and *P. taiwanensis* have been evaluated for blotch control, resulting in almost 100% pathogen and blotch inhibition (Aslani et al. 2018; Tajalipour et al. 2014).

Despite progress in methods to characterize cultivable *Pseudomonas* species, the composition of bacteria, and specifically pseudomonads, associated with mushroom caps remains unclear, as does the impact of these organisms on blotch and ultimately mushroom health and development. Determining the composition of bacteria in mushrooms with and without blotch symptoms should allow us to identify taxa that potentially impact blotch development, either positively or negatively. Moreover, because some species of *Pseudomonas* promote mushroom development while others cause disease (Osdaghi et al. 2019), it is important to specifically target pathogenic pseudomonads without inhibiting those that modulate disease control and/or mushroom development. To our knowledge, no published research has compared approaches for examining the microbiome associated with *A. bisporus* tissues. Understanding the impact of different sampling methods for assessing mushroom cap-associated bacterial composition will aid researchers in understanding the intermicrobial dynamics underlying mushroom blotch emergence or its prevention. Moreover, information about the bacteria present on asymptomatic and symptomatic mushrooms would allow us to differentiate the species present in each of the two environments as potential organisms for use in biological control.

In this project, we collected mushrooms with and without blotch symptoms from a commercial organic farm in Pennsylvania and characterized bacterial composition using three culture-independent sampling methods, followed by sequencing of the amplified 16S rRNA gene region from extracted DNA. We aimed to determine (i) which sampling approach best differentiates bacterial assemblages associated with symptomatic and asymptomatic mushrooms, and (ii) whether specific bacteria are consistently associated with mushrooms with different visible health status.

MATERIALS AND METHODS

Mushroom sampling, processing, and bacterial extraction. Mushrooms were collected from an organic mushroom farm located in Blandon, Pennsylvania, U.S.A. A total of 100 mature mushrooms were collected: 50 mushrooms demonstrated blotch symptoms and 50 were asymptomatic. This experiment was conducted once. All mushrooms collected were located in the same room, thus sampled under same the environmental conditions (ca. 17°C; CO₂ minimum 1,500 ppm, maximum 1,600 ppm). The mushrooms were collected from locations (primarily on the second bed from the floor) where blotch was observed, and asymptomatic and symptomatic mushrooms were always collected in pairs, with a distance of no more than 60 cm from each other. For each pair collection, healthy mushroom was collected first and gloved hands were sanitized with 70% ethanol between the harvesting of each mushroom. Individual

mushrooms were placed in sterile 118-ml specimen cups, closed with a screw lid and labeled. The cups containing samples were placed in a container with ice and transported to the Department of Plant Pathology and Environmental Microbiology at Pennsylvania State University in University Park, Pennsylvania, where sample processing was performed.

In order to evaluate different components of the mushroom cap microbiome, one of three methods were used to collect, respectively, lightly adhering surface-associated bacteria (“washate”), bacteria within or tightly adhering to mushroom caps (“post-washate”), or the entire community (“whole”). Initial collection involved either sonication (washate), maceration after sonication (post-washate), or direct maceration of unwashed mushrooms (whole), each followed by downstream DNA extraction (Fig. 1). All mushroom caps were initially weighed. For washate sampling, 80 ml of 10 mM phosphate buffer (pH 7.0) was added to surround each designated mushroom cap, and samples were sonicated for 5 min at 40 kHz in an Ultrasonic bath (Branson Ultrasonic Cleaner, Model 8800, Branson Ultrasonics Corp., Danbury, CT, U.S.A.). The resulting washate was used for DNA extraction. Sonicated mushrooms were then transferred to sterile stomacher plastic bags with 25 ml of 10 mM phosphate buffer (pH 7.0) and macerated for 30 s using the Seward Stomacher Model 400C Circulator. The macerated mushrooms were then used for DNA extractions (post-washate). For 50 mushrooms (25 symptomatic and 25 asymptomatic), the washate and post-washate samples were obtained from the same mushroom. For a separate 50 mushrooms (25 symptomatic and 25 asymptomatic), whole mushrooms were directly macerated in 25 ml of 10 mM phosphate buffer (pH 7.0), as described previously, with no prior sonication, and the resulting biomass was stored for DNA extractions (whole method).

DNA extractions for the washate were performed using the MoBio PowerWater DNA Extraction kit (Hilden, Germany). The washate resulting from sonication was transferred onto a 0.2 µm filter (Pall Life Sciences, #4803) by vacuum and the walls of the collection filter vessel were washed with 60 ml of sterile buffer. The filters were removed with sterile forceps and extracted according to the manufacturer’s directions. DNA extractions for samples obtained through the other two collection methods were extracted using Nucleospin 96-Soil Kits (Macherey-Nagel, Bethlehem, PA, U.S.A.). The samples containing macerated mushroom tissue resulting from the post-washate and whole methods were directly transferred into the extraction tubes (~100 mg) and further extracted as suggested by the manufacturer.

PCR amplification and sequencing. A two-step amplification and dual-barcoding approach (Kozich et al. 2013) was performed to generate Illumina-compatible amplicons (R. Trexler and T. Bell, *unpublished data*). Specifically, we used the universal bacterial primer set 515F (5′-GTGYCAGCMGCCGCGGTAA-3′) and 806R (5′-GGACTACNVGGGTWTCTAAT-3′) to amplify the v4 region of the 16S rRNA gene (Apprill et al. 2015; Parada et al. 2016). PCR conditions were as follows: 8 µl of 5Prime HotStart MasterMix (Quanta BioSciences Inc., Beverly, MA, U.S.A.), 0.1 to 1 µl of template DNA, 1 µl of each primer from 10 µM stocks, and the appropriate volume of molecular biology grade water to bring the reaction to 20 µl. Thermal cycling was carried out on Mastercycler Nexus Gradient PCR machines (Eppendorf, Hamburg, Germany) under the following conditions: 94°C for 3 min; 25 cycles of 94°C for 45 s, 50°C for 60 s, and 72°C for 90 s; followed by a final elongation for 10 min at 72°C.

The resulting amplicons were cleaned using Mag-Bind TotalPure NGS (Omega Bio-Tek, Norcross, GA, U.S.A.) magnetic beads. The target gene primers were designed with overhang regions for a secondary PCR to attach the barcodes and standard Illumina

adapters, and accordingly 5 µl of cleaned PCR product, 12.5 µl of 5Prime Hotstart Mastermix, 2.5 µl of water, and 2.5 µl of each index primer (10 µM) were added to each reaction and amplified under the following conditions: 98°C for 1 min; eight cycles of 98°C for 15 s, 55°C for 30 s, and 72°C for 20 s; followed by a final elongation for 5 min at 72°C. Barcoded amplicons were then normalized to 25 ng/sample using the SequalPrep Normalization Plate Kit (Invitrogen, Carlsbad, CA, U.S.A.), pooled, and concentrated on a Savant SpeedVac (Thermo Scientific, Waltham, MA, U.S.A.) at 50°C for 3 h. Half of the pool (12.5 ng/sample) was then purified via agarose gel extraction with the PureLink Quick Gel Extraction kit (Invitrogen) and sent to the Cornell University Biotechnology Resource Center Genomics Facility where it was sequenced on an Illumina MiSeq using the 2 × 250 cycle v2 kit according to the manufacturer's recommendations. After quality filtering, we obtained 10,008,086 total reads across 148 samples, providing an average of 67,622 reads/sample. Raw read data were submitted to the NCBI SRA under the accession number PRJNA532639.

Initial data processing. Read processing and data analysis was completed (Howard et al. 2017) (R. Trexler and T. Bell, *unpublished data*). Here, Mothur v1.39.5 (Schloss et al. 2009) was used to merge forward and reverse reads and trim primer sequences (pdiffs = 2, maxambig = 0). After removing singletons, reads were split into single fastq files for each sample and the appropriate qiime labels were added using MacQIIME v1.9.1 (Caporaso et al. 2010) before concatenating back into a single data file. A modified Brazilian Microbiome Project pipeline was utilized for operational

taxonomic unit (OTU) clustering (Pylro et al. 2014). Specifically, USEARCH 7 (Edgar 2010) was used to dereplicate sequences and cluster OTUs at 97% similarity. OTU taxonomy was assigned in Mothur using the greengenes May 2013 database (McDonald et al. 2012) and OTUs classified as Archaea, Eukaryota, Plantae, chloroplast, mitochondria, and 'unknown' were removed from the dataset.

Further analysis was performed on the R software (version 3.6.1). Samples were rarefied to 1900 reads/sample and those falling below this threshold were removed from the dataset. Bray-Curtis dissimilarities, nonmetric multidimensional scaling, alpha diversity measures, and permutational analysis of variance tests were performed using the 'vegan' R package (Oksanen et al. 2015). Community composition was analyzed using the 'phyloseq' (McMurdie and Holmes 2013) and 'metacoder' (Foster et al. 2016) R packages. For the metacoder R package, OTUs of the bacterial community between symptomatic and asymptomatic mushroom caps were compared, considering all the bacterial extraction methods. Differences in taxonomic distributions between extraction methods were tested using two-sided Student's *t* test with the 'stats' R package. Graphs and figures were further refined using Adobe Illustrator CC 2018.

Comparison of sequences to *Pseudomonas* type strains and reference strains for unnamed *Pseudomonas* spp. Because *Pseudomonas* spp. are important members of the mushroom microbiome and devome, we attempted to more finely identify *Pseudomonas* spp. in these samples using exact sequence variants

Fig. 1. Scheme of the methods for the isolation of bacteria from asymptomatic and symptomatic mushrooms (*Agaricus bisporus*) collected from an organic farm located in Pennsylvania.

(ESVs). For the purpose of including sequences classified as *Pseudomonas* in a phylogenetic tree alongside cultured representatives, we reprocessed our data using the DADA2 pipeline (Callahan et al. 2016) to ESVs. We primarily followed the DADA2 Pipeline Tutorial 1.6: https://benjjneb.github.io/dada2/tutorial_1_6.html. ESVs identified as *Pseudomonas* spp. were compared with 162 *Pseudomonas* spp. type strains and reference strains of 120 putative *Pseudomonas* spp. published previously (Hesse et al. 2018) and 27 previously cultured blotch-causing bacteria isolated from mushroom (S. Martins et al., *unpublished data*).

After assessing forward and reverse read quality (command ‘plotQualityProfile’), we trimmed our sequences and removed primers with the command ‘filterAndTrim’ using the following parameters: truncLen = c(240,160), trimLeft = c(19, 20), maxN = 0, maxEE = c(2,2), truncQ = 2, rm.phix = TRUE, compress = TRUE, multithread = TRUE. We then determined the run-specific error rate with the command ‘learnErrors’, dereplicated sequence (command ‘derepFastq’), and used the DADA2 algorithm to infer true sequence variants in forward and reverse reads (command ‘dada’). Forward and reverse reads were then merged (command ‘mergePairs’) and assumed chimeras removed (command ‘removeBimeraDenovo’, method = “consensus”). Taxonomy was assigned with the command ‘assignTaxonomy’ using the Silva database v.128 (silva_species_assignment_v128.fa) (Quast et al. 2012).

To determine which ESVs that classified as *Pseudomonas* were significantly different in relative abundance between symptomatic and asymptomatic mushrooms, we performed differential abundance analysis using the DESeq2 (v.1.23.10) and phyloseq (v.1.27.6) R packages (Love et al. 2014; McMurdie and Holmes 2013, 2014). These analyses were performed on sequence data that had not been normalized through subsampling.

First, relative abundance data, taxonomic data, and metadata were combined into a phyloseq object. We then separated the object by sampling method (i.e., wash, mushroom interior, and whole mushroom), and performed downstream DESeq2 analyses

separately for each method. We used the ‘phyloseq_to_deseq2’ command followed by the ‘DESeq’ command (test = “Wald”, fitType = “parametric”) to test for ESVs that differed significantly in relative abundance between symptomatic and asymptomatic mushrooms. We then subselected ESVs with an adjusted *P* value less than 0.01, and extracted only those classified as *Pseudomonas*. These were then used to generate and annotate the phylogenetic tree, using neighbor joining algorithm and general time reversible for the nucleotide substitution model. Branching values were determined using 1,000 bootstraps and *E. coli* NCTC9001^T was used as outgroup using CLC Genomics Workbench 11 (Venlo, The Netherlands).

RESULTS

There were significant differences in bacterial composition depending on the health status of the mushrooms (*P* = 0.001) and sampling method (*P* = 0.009) (Fig. 2A). Within the mushroom cap, *Proteobacteria* and *Bacteroidetes* were the most abundant bacterial phyla regardless of extraction method or mushroom health status (Fig. 2B). No significant differences were detected in the interaction between health status and sampling method (*P* = 0.929) (Fig. 2A). Thus, we discuss the main effects (sampling method or health status) in separate sections below.

With regards to alpha diversity, no significant interactions were observed between sampling method and mushroom health based on Shannon diversity (*P* = 0.7320), OTU richness (*P* = 0.7940), and Pielou’s evenness (*P* = 0.7498). Among the three bacterial sampling methods, washate and post-washate exhibited the highest Shannon diversity (*P* = 0.027) and Pielou’s evenness (*P* = 0.0359) (Fig. 3AC). However, no significant differences were detected for observed OTU richness (*P* = 0.312) (Fig. 3B). When we compared diversity metrics based on mushroom health status, we observed higher Shannon diversity (*P* < 0.001), OTU richness (*P* < 0.001), and Pielou’s evenness (*P* = 0.004) in asymptomatic mushrooms (Fig. 3A to C).

Fig. 2. A, Nonmetric multidimensional scaling (NMDS) of global 16S rRNA gene composition from symptomatic and asymptomatic *Agaricus bisporus* extracted through different methods (washate, post-washate, and whole). **B**, Relative abundance of operational taxonomic units at the phylum level.

Overall difference between asymptomatic and symptomatic mushroom microbiome. As stated previously, differences were observed in bacterial composition depending on the health status of the mushroom cap ($P = 0.001$). The relative abundance of most bacterial families was comparable in both symptomatic and asymptomatic mushrooms (Fig. 4, branches and nodes in gray). However, several families were more abundant in asymptomatic mushrooms (branches and nodes colored in cyan). In contrast, only a few families, including *Enterobacteriaceae*, *Sphingobacteriaceae*, *Flavobacteriaceae*, and *Weeksellaceae*, were more abundant in or on symptomatic mushrooms (branches and nodes colored in red).

Comparison between the methods (washate and post-washate mushroom) and mushroom health status. We compared washate and post-washate bacterial composition from the same individual mushroom caps because these samples together should encompass the entire mushroom cap microbiome, and because they potentially represent two distinct components of the microbiome: bacteria that are easily removed from the cap by sonication (washate) and bacteria that remain associated with the mushroom after sonication and are potentially internal to the cap (post-washate). For both symptomatic and asymptomatic mushrooms, most taxa were equally abundant in both microbiome components (Fig. 5A branches and nodes in gray). However, there were more significantly overrepresented taxa in the tightly adhering/internal component (post-washate, shown in green) than in the component that is easily removed by sonication (washate), regardless of health status. For instance, there were 14 and 36 more overrepresented taxa tightly adhering to the mushrooms for symptomatic (Fig. 5B) and asymptomatic (Fig. 5C) mushrooms, respectively. Among the *Proteobacteria*, the most abundant phylum for all treatments (Fig. 2B), the *Gammaproteobacteria* were overrepresented in the lightly adhering component (washate, $P > 0.05$), while *Alphaproteobacteria* were overrepresented in the tightly adhering/internal component (post-washate, $P > 0.001$) (Fig. 5C).

Interestingly, particular taxa differed with respect to whether they were overrepresented in one of the microbiome components depending on mushroom health status. For instance, regardless of visible mushroom health status, the phylum *Acidobacteria* was

more abundant in the lightly adhering component represented by the washate method (shown in brown). In contrast, the phyla *Armatimonadetes* and *Gemmatimonadetes* as well as the classes *Spartobacteria*, *Pedospaerae*, and *Opitutae* from the phylum *Verrucomicrobia* were only overrepresented in the tightly adhering/internal component (post-washate) and only from asymptomatic mushrooms (Fig. 5B and C). Likewise, the families *Chthoniobacteraceae* and *Opitutaceae* were also overrepresented in asymptomatic mushrooms but only in the lightly adhering component (washate, Fig. 5C, shown in brown).

Globally, the genus *Pseudomonas* was overrepresented by the washate method (Fig. 5D). Because of this and the importance of the genus to mushroom development and health, we further analyzed the exact sequence variances (ESVs) from the genus *Pseudomonas*. Most of the ESVs were distributed evenly among all of the samples, regardless of health status or microbiome component (Fig. 6). Four ESVs (ESV565, ESV1109, ESV221, and ESV1008) were most closely related to the mushroom pathogen *P. agarici* (Fig. 6). This was not the case for all blotch pathogens however. For example, strains isolated from Pennsylvania mushroom houses and previously identified as *P. tolaasii* (S. Martins et al., *unpublished data*; BP1104, BP1106, BP1199, BP1210, BP1243, BP1271, and BP1242) were all within a large clade that included the type strain of *P. tolaasii* and several other type strains of fluorescent pseudomonads. There were two ESVs in this clade, but we cannot confirm whether or not they truly represent *P. tolaasii* strains in these samples. Sequences for other unnamed Pennsylvania blotch pathogens (S. Martins et al., *unpublished data*) were nearly identical to some of the ESVs detected (e.g., BP1286 and ESV31).

DISCUSSION

The long-term goal of our research is to understand the microbial factors influencing spatiotemporal dynamics of bacterial blotch pathogens from the genus *Pseudomonas* on mushroom (*A. bisporus*) caps and disease development with the goal of manipulating these pathogens to increase commercial mushroom yield and quality. Results from a variety of studies using culture-dependent methods lead researchers to hypothesize that the mushroom cap

Fig. 3. Comparison among bacterial isolation methods (washate, post-washate, and whole) and between mushroom (*Agaricus bisporus*) health status (asymptomatic and symptomatic). **A**, Shannon diversity; **B**, operational taxonomic unit (OTU) richness; and **C**, Pielou's evenness. Among the methods, bars with the same lowercase letter are similar at the 5% level according to Tukey's test. Between mushroom health, ** indicates significant at the 0.01 probability level; and *** indicates significant at the 0.001 probability level. ns = not significant.

authors found that bacteria were the more abundant compared with yeast and fungi, this study concentrated exclusively on bacterial composition. In order to understand what influence bacterial succession, and in particular pathogen succession is having on mushroom health status, it was necessary to first determine whether there is a difference in microbiome composition on mushroom caps that are visibly observable as either symptomatic or asymptomatic. A second reason for concentrating on bacteria was that it helps to characterize potential relationships between blotch pathogens and interactions with other bacteria.

The data presented here demonstrate that bacterial composition differed between symptomatic and asymptomatic mushrooms (Fig. 2A), with higher bacterial diversity observed in asymptomatic rather than symptomatic mushrooms (Fig. 3A). For example, there were 14 and 36 more overrepresented taxa internal or tightly adhering to the mushrooms for symptomatic (Fig. 5B) and asymptomatic (Fig. 5C) mushrooms, respectively. Particular families within the phyla *Planctomycetes*, *Acidobacteria*, *Gammatimonadetes*, *Actinobacteria*, *Firmicutes*, and *Verrucomicrobia* were responsible for this overrepresentation (Fig. 4). Barring artifacts due to overrepresentation of individual taxa in the symptomatic mushrooms, the overrepresented taxa from asymptomatic mushroom caps will serve as targets for future research to determine whether they are involved in inhibiting blotch pathogens and reducing the incidence or severity of disease on mushroom caps.

Alternatively, the significantly lower diversity (Fig. 3A), richness (Fig. 3B), and evenness (Fig. 3C) of bacteria in symptomatic compared with asymptomatic mushrooms might be explained by suppression of other bacteria by the dominant pathogens (e.g.,

Pseudomonas) associated with symptomatic mushrooms. For instance, *Pseudomonas* spp. produce active proteinases and lipases, which increase fitness against other microorganisms in the *A. bisporus* system (Chung et al. 2014). Likewise, *P. tolaasii* produces volatile organic compounds (Lo Cantore et al. 2015), which could also inhibit the growth of other microbes in symptomatic mushrooms, due to the broad spectrum of these compounds (Martins et al. 2019; Terra et al. 2018). We have preliminary data that indicate that blotch pathogens produce compounds that inhibit each other and other *Pseudomonas* species (R. Hershlag et al., unpublished data). Antibiosis by *Pseudomonas* spp. may also be responsible for the underrepresentation of certain taxa (e.g., *Planctomycetes*, *Acidobacteria*, *Gammatimonadetes*, *Actinobacteria*, *Firmicutes*, and *Verrucomicrobia*) on symptomatic mushrooms.

Some taxa, such as the families *Enterobacteriaceae* and *Sphingobacteriaceae*, were more abundant in symptomatic mushrooms (Supplementary Table S1). It is not known whether these organisms are overabundant because they are not inhibited by *Pseudomonas* spp. and can colonize at higher densities due to the lack of competitors. Alternatively, the correlated organisms may interact synergistically with blotch pathogens. For example, members of family *Sphingobacteriaceae* produce catalase and oxidase (Lambiase 2014), which may be involved in cell wall degradation in mushroom. However, there is no published link between the presence of bacteria from the families *Enterobacteriaceae* and *Sphingobacteriaceae* and blotch in mushroom.

The second significant advance from this work is the finding that sampling approach can impact the resulting description of the

Fig. 5. Differential heat tree showing differences in bacterial composition to the family level. **A**, The gray tree on the left functions as a key for the smaller unlabeled trees. The comparisons were made among the methods of bacterial isolation post-washate and washate from **B**, symptomatic and **C**, asymptomatic mushrooms (*Agaricus bisporus*). For each taxon, a Wilcoxon rank sum test was used to test for differences between the median abundances of samples in each treatment. Taxa in brown are statistically more abundant in the washate method, while those in green are statistically more abundant in the post-washate method. The branches indicate the association between taxa and the node sizes indicate the operational taxonomic unit (OTU) counts per taxon. **D**, Comparison between washate and post-washate methods and resolving asymptomatic and symptomatic for *Pseudomonas* spp. *Significant at the 0.05 probability level by Student's *t* test.

bacterial composition associated with mushrooms caps (Figs. 2A, 3A and C, and 5). Compared with the washate/post-washate methods, the whole mushroom did not differ regarding the two most abundant phyla, *Proteobacteria* and *Bacteroidetes* (Fig. 2B). However, a lower Shannon diversity and Pielou's evenness were found for the bacteria present in samples when the whole mushrooms were sampled compared with the samples where the loosely or tightly adhering bacteria were analyzed separately (Fig. 3). The

combining of the loosely or tightly adhering bacterial fractions in the whole microbiome samples appears to quench the signals from less abundant bacterial sequences in either fraction because both Shannon diversity and Pielou's evenness respond to abundance. These sampling protocols can now be used to strategically target given populations. For example, more *Pseudomonas* spp. were found in the washate of symptomatic and asymptomatic mushrooms. This diversity largely represents the overall *Pseudomonas*

Fig. 6. Comparisons of exact sequence variants (ESVs) to type and reference strains from known *Pseudomonas* spp. The node, text, and branch of 27 cultured blotch-causing bacteria isolated from mushroom (*Agaricus bisporus*), *Pseudomonas* type strains, and the ESVs are labeled, respectively, in red, blue, and green. The asterisks (*) before the bacterial names indicate type strains of known blotch-causing agents. Neighbor joining algorithm and general time reversible were used for the nucleotide substitution model. Branching values were determined using 1,000 bootstraps. *Escherichia coli* NCTC9001T was used as outgroup.

composition of the mushroom cap and could be used as a surrogate for the whole cap. Additionally, pathogenic *Pseudomonas* species causing blotch colonize the mushroom surface tissues prior to initiating disease (Saxon et al. 2014). Alternatively, it may be strategic to use post-washate samples to study or isolate bacteria other than pseudomonads for biological control because of their association with asymptomatic mushrooms. For example, families in *Bacillales*, *Bdellovibrionales*, and *Xanthomonadales* were among the 33 taxa that were more abundant in the internal or tightly adhering component of asymptomatic mushroom microbiomes, and members of these families have been reported to provide biocontrol responses against blotch in mushroom (Aslani et al. 2018; Saxon et al. 2014; Tajalipour et al. 2014).

Bacteria in the *Proteobacteria* and *Bacteroidetes* are a dominant feature in mushroom production. Like other research on *A. bisporus* (Godfrey et al. 2001; Siyoum et al. 2016; Yang et al. 2019) and wild mushroom (Venturini et al. 2011) cap microbiomes, we observed that mushroom cap microbiomes were predominantly composed of sequences from members of *Proteobacteria* and *Bacteroidetes* (Fig. 2B). This work demonstrated for the first time that *Proteobacteria* and *Bacteroidetes* were consistently distributed regardless of bacterial isolation method or mushroom health status (Fig. 2B). It is hypothesized that the caps are colonized with bacteria from the growth substrate. For example, a peat moss-based casing layer showed a higher relative abundance of *Proteobacteria* and *Bacteroidetes* (Pecchia et al. 2014). However, the dominant bacteria in the compost differ from those found in the mushroom caps and casing layer. Firmicutes comprise the majority of bacteria prior to the introduction of *A. bisporus* inoculum to compost (Vieira and Pecchia 2018).

The bacterial profiles of *A. bisporus* mushroom caps were primarily composed of *Pseudomonas* spp. (~60% of the total sequences in this study), i.e., members of *Proteobacteria* (Fig. 2B), which is in accordance with other studies (Godfrey et al. 2001; Siyoum et al. 2016; Yang et al. 2019). In developing strategies for microbiome manipulation and reducing pathogen populations, it is important to remain cognizant that members of the genus *Pseudomonas* are important members of the mushroom devome (organisms and outcomes of microbial mediated development). Thus, adequate sequence-based taxonomic or pathogen-specific markers and techniques are needed to differentiate pathogenic from beneficial *Pseudomonas* spp. or strains. The research reported here coupled to culture-dependent methods, which circumscribed the diversity of pathogens present in Pennsylvania mushroom farms (S, Martins et al., *unpublished data*) and sequencing of type strains of 166 species or subspecies and identification of reference strains for an additional 189 unnamed species of *Pseudomonas*, serves as the first proof of concept for ESV diagnostics for bacterial blotch of mushrooms or other diseases. The data indicate that this method was predictive for previously identified *P. agarici* strains. Six ESVs were most closely related to the same region in the mushroom pathogen *P. agarici* and other blotch pathogens from Pennsylvania. Additionally, sequences for other unnamed Pennsylvania blotch pathogens were similar to detected ESVs (e.g., BP1286 and ESV21). Although, isolation of strains with these ESVs from these samples followed by testing of pathogens from these communities would provide final confirmation of the presence of these pathogens in the samples with these ESVs, it appears that ESVs from this 16S rRNA gene fragment may be diagnostic for some pathogens.

Previously, *P. protegens*, a soilborne organism used for biological control of soilborne fungal pathogens of plants, was shown to cause mushroom browning and discoloration similar to symptoms produced by blotch pathogens (Henkels et al. 2014). To our knowledge, it has never been isolated from mushrooms with blotch from a commercial mushroom facility or elsewhere. The data presented

here indicate that *P. protegens* was not present in or on the mushrooms sampled as none of the ESVs were closely related to *P. protegens*.

It is likely that the ESV approach with this sequence fragment is not diagnostic for the most important blotch pathogen, *P. tolaasii*. The DNA sequences of this fragment for previously identified *P. tolaasii* strains (S. Martins et al., *unpublished data*) encompass 14 different named species including *P. tolaasii*. Some ESVs from the samples evaluated in this research were grouped close to the pathogens identified in our previous study, but it is not clear whether they are likely to represent *P. tolaasii* or other *Pseudomonas* spp. Other genes and sequence fragments are being explored that are likely to provide better resolution for *P. tolaasii* and other blotch-causing *Pseudomonas* spp. (Aleman et al. 2019).

This research made strides in describing the mushroom cap microbiome and established sampling methods that will allow different segments of the bacterial community to be targeted including pathogens in the genus *Pseudomonas* and organisms with potential for biological control. Although the ESV diagnostic approach may be sufficient for some pathogenic species (*P. agarici*), it is clear from this work that sequences from fragments of other genes will be needed to detect the most important bacterial blotch pathogen (*P. tolaasii*) from mushroom and environmental samples.

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