INVESTIGATION INTO THE MICROBIAL COMMUNITY CHANGES THAT OCCUR IN THE CASING LAYER DURING CROPPING OF THE WHITE BUTTON MUSHROOM, *AGARICUS BISPORUS*

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ABSTRACT

Decrease in yield occurs after each subsequent flush, or break, of the commercially produced white button mushroom, *Agaricus bisporus*. The exact cause behind the reduced yield is unknown, though there are several theories. One theory is that chemical byproducts (e.g. salt crystals) form on the mycelium thereby reducing nutrient uptake and subsequent fruiting. A second theory is that the nutrition of the substrate is reduced throughout the cropping process thereby limiting yield potential and subsequently reducing the yield of each break. Another explanation for the reduced yield may be related to the diverse microbial communities present in either the casing layer or the compost substrate itself. It is believed that certain microbial communities are needed and are responsible for promoting primordial formation and maturity. This study was an investigation into the differences that occurred in the major bacterial groups present in the casing layer during cropping. The casing layer consisted of a sphagnum peat moss buffered with calcium carbonate that was added to the compost after a 17 day spawn run. Samples of the casing layer from an *A. bisporus* crop were collected on days 6, 13, 22 and 29 (days post-casing) during the production period. DNA was extracted from the replicate casing samples and bacterial DNA was amplified using PCR and then isolated from each sample for metagenomic analysis. Statistically significant changes in populations of Actinobacteria, Firmicutes, Bacteroidetes, and Proteobacteria were observed, with minor changes also seen in Chloroflexi, Gammatimonadetes, and Planctomycetes. A better understanding of the microbial communities in the compost and casing will hopefully allow us to increase the bioefficiency during production as well as possibly help us to better understand microbial community-pathogen relationships as they relate to disease development.

Keywords: casing, phylogenetics, microbial population, *Agaricus bisporus*

INTRODUCTION

In 2013, the US produced approximately 882 million pounds of *Agaricus bisporus*, the white button mushroom, valued at $1.05 billion USD (USDA) [1]. Profitability continues to be a challenge to US mushroom growers because of increased cost of production due to increase in raw material, energy and labour expenses. Most US producers harvest mushrooms for three breaks, (flushes) with the highest yield occurring during the first break and declining yields during subsequent beaks. In an attempt to explain this phenomenon three major hypotheses have emerged. Beyer and Mutherbaugh [2], proposed that depletion of compost nutrients over a cropping period may limit future yields. Beyer also supports Schisler's hypothesis that an accumulation of toxic metabolites may inhibit mushroom growth [2]. Zarenejad *et al*. [3], assert that *A. bisporus* yields are affected by changes in the casing microflora that may change during cropping and consequently inhibit fruiting.

The aforementioned hypotheses are not mutually exclusive; therefore, it is likely that the yield decrease as a product of many complex interactions. Although previous studies, including those conducted by Hayes *et al.* [4], have shown that the bacteria in the casing layer exhibit growth-stimulating activity, the majority of research into *A. bisporus* cultivation has focused on compost preparation. For example, Gerrits produced a detailed list of thermophilic fungi and actinomycetes that he suggests are important organisms, along with temperature ranges for optimal activity of each species [5]. Schisler also described specific thermophiles required for normal compost preparation, which includes ammonifying bacteria from the genera *Proteus*, *Micrococcus* and *Aerobacter* [6].

To address the dearth of research, this study will serve as an initial survey of the major bacterial phyla present in the casing layer. Significant changes in the sizes of their populations over a typical harvesting period will also be assessed. Future research investigating these changes may lead to solutions for more efficient mushroom cultivation, higher yields, decreased disease pressures and subsequent increased profitability for growers of *A. bisporus*.

Before the cause of reduced yields can be investigated, it is important to understand the cropping process and the role of casing microbiota in commercial *A. bisporus* production. First, compost consisting largely of horse manure is prepared to serve as the growth medium [7]. Meticulous preparation of composting material is vital, as it provides nutrients for growth of both mushrooms and associated microflora. These nutrients include: sources of nitrogen, commonly supplied through chicken manure, brewer's grains, and/or cottonseed meal [7] complex carbohydrates, such as cellulose and lignin; water; vitamins; and minerals [5]. Developing the ideal substrate for *A. bisporus* is complicated by the obligate co-existence with microbes. Besides the fact that they also require nutrients for growth and metabolic processes, microbiota with that may either stimulate or inhibit fruiting may require additional factors for which is still unspecified [4]. While inherently difficult to achieve, the ideal compost provides a stimulatory environment for development of both the mushrooms and symbiotic microflora along with inhibiting growth of pathogens or competitors [7]. By investigating changes in casing bacterial population and understanding the roles of major groups in the growth of *A. bisporus*, the task of developing an ideal substrate will be more easily attainable.

MATERIALS AND METHODS

Cropping

Compost was prepared at the Mushroom Research Center (MRC) at The Pennsylvania State University following standard methods, with Phase I taking place in forced aerated bunkers. The standard composting formula consisted of straw-bedded horse manure, switch grass straw, poultry manure, dried distillers grain and gypsum. The aerated Phase I composting period lasted 6 days and was turned on day 3 and filled on day 6 into wooden trays and moved into a Phase II room at the MRC. Temperatures were maintained according to MRC standards for 8 days and pasteurization and conditioning were performed. After 8 days in Phase II, the substrate was spawned with a commercial off-white hybrid strain at a rate of 2.5% and supplemented with a commercial supplement at the manufacturer's recommended rate. 22.7 kg (50 lbs.) of wet substrate was layered into 0.26 m^2 (2.75 ft^2) plastic growing tubs and the substrate in each tub was pressed tightly with a hydraulic press. Fifty four tubs were placed on metal racks and moved to an environmentally controlled production room at the MRC where temperature and humidity were maintained for the duration of the 17 day spawn run period.

Standard MRC casing consisted of sphagnum peat moss mixed with agricultural limestone added @ 22.7 kg limestone/ 0.17 m^3 peat (50 lbs./ 6 ft^3) with a commercial casing inoculum (CI). The room was flushed with fresh air and temperature lowered 5 days after casing to initiate primordial development. First break harvest began 10 days after the fresh air flush and 16 days after casing. Mushrooms were harvested and yield data recorded for 3 growing cycles or breaks.

Sample collection and preparation

Casing layer samples were collected from the *A. bisporus* crop (MRC 1303) at the MRC. Samples of the casing layer were obtained at four different periods over the cropping cycle: day 6, 13, 22, and 29. Day 6, or the sixth day after casing, is when primordial formation typically occurs. Day 13, 22, and 29 after spawning represent the onset of the first, second, and third flush, respectively. On each sampling date, a #10 plug core sampler was used to obtain 3 plugs of the casing layer from 3 separate tubs, totaling 9 samples. The plugs were then combined into a pooled sample and stored at -20°F. This process was repeated twice more to provide a total of 3 pooled casing samples for each date. Frozen samples were then ground in liquid nitrogen prior to extracting DNA. DNA was extracted using the MoBio PowerSoil® DNA Isolation Kit. DNA extractions were carried out three times from each pooled sample. Extracted DNA was subsequently amplified for pyrosequencing using the 16S rRNA universal primers 27F (5'-AGAGTTTGATCMTGGCTCAG–3') and 907R (5'-CCCCGTCAATTCMTTTGAGTTT-3').

Metagenomic and statistical analysis

Metagenomic analyses of the extracted DNA were conducted at the Penn State Bioinformatics Consulting Center. The three pooled samples representative of each collection date were sequenced individually, and the results were combined into a single set. Using MEGAN, statistical comparisons between metagenomic data sets were completed to determine significant changes in bacterial populations during a cropping period. Default parameters were used to assess the lowest common ancestry (abbrev. LCA) among bacteria, with the exception of the minimum probability value. This was set to 0.95 to exclude matches with less than 95% certainty of being correctly identified. Statistical comparisons were carried out as described by Suparna Mitra *et al*. [8] using a critical *p*-value of 0.01. The corrected Holm-Bonferroni method was utilized to compare the following sets of data: Day 6 vs. 13; Day 13 vs. 22; and Day 22 vs. 29.

RESULTS

Fig. 1 provides a comprehensive list of the bacterial phyla identified in the casing samples taken on the four sampling dates. Actinobacteria, Bacteroidetes, Firmicutes, and Proteobacteria were found to be the four predominant phyla of casing microbiota. As shown in Table 1, the populations of both Actinobacteria and Firmicutes decreased significantly after Day 6 but increased on Day 22 and 29. Levels of Bacteroidetes present in the casing layer increased over the cropping period until Day 22, with a subsequent decrease on Day 29. Proteobacteria, the largest bacterial population identified in the samples, showed an increased population on Day 13, which was followed by decrease on Day 22 and 29. Populations of the Phylum Gemmatimonadetes and Planctomycetes increased significantly with each sampling date. The population of Chloroflexi increased significantly from day 6 to day 13 with a significant drop in numbers on day 22 and 29. These populations are not included in Table 1 due to their relatively minor contributions to the overall population size (each making up less than one percent of the total reads); however, these three groups exhibited significant changes in their populations. Interpretation of the ecological importance that the major 4 phyla identified in the casing layer has is a difficult undertaking. To have better understading on what role these phyla have in the casing layer, the significant orders identified with each group are listed in Table 2, according to comparative population size.

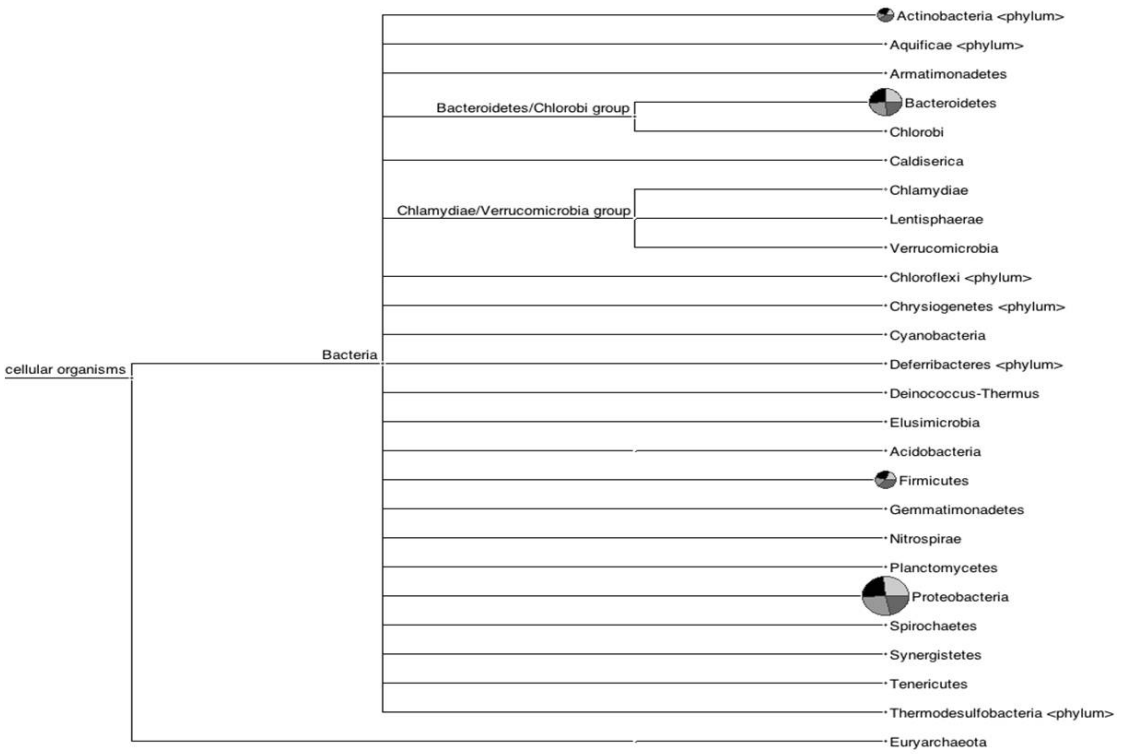

Figure 1. Taxonomy profile of casing microbiota. Comprehensive list of bacterial phyla found in casing samples from Day 6, 13, 22, and 29. Size of the circle preceding a group is representative of its relative population size. The four major groups identified were as follows: Actinobacteria, Bacteroidetes, Firmicutes and Proteobacteria.

Table 1. Highlighted differences between different casing samples. "Difference" column represents the statistically significant increase (+) or decrease (-) in number of reads identified in each group

Phylum	**Day 6 to Day 13**		**Day 13 to 22**		**Day 22 to Day 29**	
	Difference	*P-value*	*Difference*	*p-value*	*Difference*	*p-value*
Actinobacteria	-	0.01	+	0.01	+	9.3×10^{-6}
Bacteroidetes/Chlorobi group	+	0.01	+	0.005	-	0.001
Firmucutes	-	0.01	+	0.01	+	0.01
Proteobacteria	+	0.01	-	0.01	-	4.6×10^{-20}

Table 2. Significant orders within major phyla. Orders that form the majority of each phylum within the casing samples

Phylum	**Significant Orders**
Actinobacteria	Actinomycetales; Coriobacteriales
Bacteroidetes/Chlorobi group	Flavobacteriales; Sphingobacteriales
Firmicites	Bacillales; Clostridiales
Proteobacteria	Rhizobiales, Caulobacteriales, Sphingomonadeles; Burkholderiales; Xanthomonadales, Pseudomonadales

DISCUSSION

In this initial survey of microbiota found in a sphagnum peat moss-based casing layer, the major groups were found to be Actinobacteria, Bacteroidetes, Firmicutes, and Proteobacteria. Actinobacteria, a phylum that includes common soil microorganisms and nitrogen-fixing bacteria, represented up to 10 per cent of the casing bacterial community. Bacteria belonging to the phylum Bacteroidetes made up approximately 25% of the community profile in the casing layer. This group includes the orders Flavobacteriales and Sphingobacteriales. Members of Flavobacteriales are able to carry out *de novo* biosynthesis of nicotinamide adeosine diphosphate (NAD), which is an essential cofactor in many metabolic pathways. These bacteria also have roles in lipid degradation which may affect the nutrition of the substrate for *A. bisporus* (NCBI). Order Bacillales, belonging to phylum Firmicutes, contains species that produce vital coenzymes. Specifically, members of this order can synthesize folate, an important precursor required for amino acid, DNA or RNA biosynthesis (NCBI).

Bacteria belonging to the phylum Proteobacteria were predominant population present in the casing, making up between 45 and 55 per cent of the bacteria profile on the different sampling dates. This phylum contains multiple species, including one that has been studied for its role in mushroom production, specifically *Pseudomonas putida*, which has been proposed to play a role in primordia maturation [9]. Previous studies have indicated that *Pseudomonas* species play a role in metabolizing volatile organic compounds, produced by *A. bisporus* that are inhibitory to primordia maturation. Hayes and Nair [10], showed that *Pseudomonas* species increased from 34% of the population at casing up to 95% of the casing population at the end of the cropping cycle. The pseudomonas are in the Gamma proteobacteria class (phylum Proteobacteria) which, in this study, demonstrated a decrease in the population of the bacterial communities from day 6 through day 29, with the greatest decline occurring between day 22 and day 29. However, the Gamma proteobacteria still made up approx. 4-5 percent of the reads from each sampling period.

The majority of the previous studies investigating microbial populations within the substrate and in the casing have often been carried out using traditional microbiological techniques (i.e. plating). This allows us to look at nutritional requirements and changes of the bacterial communities that are able to grow in culture. However, it is estimated that as few as 2% of bacterial populations in environmental samples are able to be cultured on media [11]. Previous studies are very beneficial and give us a baseline on what role certain microbial communities play in mushroom nutrition and disease development, however, there is currently an enormous void in our true understanding as to what roles these microbes may actually play

in mushroom production. To expand on the meaningfulness of the results of this study, more detailed analyses of the casing microbiota should be done to elucidate bacterial compositions down to the genus level to determine what key role they may play in mushroom nutrition and development. This could eventually lead to targeted solutions for increasing yields. This study was intended to conduct a general survey of the casing microbiota found in a sphagnum peat-based casing material, and the results were presented with an assumption that the largest bacterial populations have the greatest influence on *A. bisporus*. It is quite possible that other bacterial communities identified in this study, especially those that also exhibited significant changes over the cropping period, play important roles in the growth and development of *A. bisporus* despite of their relatively small makeup of the total bacterial community. Furthermore, an investigation into the fungi present in the casing layer should also be made to determine what role these organisms play in *A. bisporus* production and disease development. Composition of the microflora is likely to vary with modification of compost and casing materials. Much more research is needed to better understand how these variables affect the potentially beneficial microbial communities present in the mushroom production system.

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