The Population Dynamics of Various Pseudomonas Pathogens in Different Mushroom Types

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Abstract

Bacterial blotch is a complex of diseases that results in spotting and discoloration in a variety of mushroom species. In Pennsylvania and other US regions, it is one of the most important diseases to study due to its wide impact on commercial mushroom production. As of now, we know that at least 11 species of *Pseudomonas* cause blotch disease in Pennsylvanian mushrooms. In this study, we are researching whether the virulence of previously identified blotch pathogens remains consistent on a variety of cultivated mushrooms over time. We want to determine whether there are differences in the virulence of these *Pseudomonas* strains on one type of mushroom and whether a certain strain is more virulent than the other. The goal of this research is to increase our understanding on the mechanisms of these pathogens so that management strategies can be improved, thus reducing the impact of disease on the yield and quality of commercial mushrooms. Four different mushroom varieties were inoculated with four different Rifampicin resistant strains and a control buffer. Pathogenicity tests were conducted to collect data for bacterial growth, which was analyzed through statistical distribution. The results indicated susceptibility of the Oyster and King Bolete mushrooms to all the *Pseudomonas* strains, as they allowed growth of bacteria in the tissue. On the other hand, both the Maitake and Shiitake varieties, recognized medicinal mushrooms, were not susceptible and displayed no physical characteristics of infection. This raises questions about the potential of utilizing these medicinal mushrooms as a treatment for blotch disease in other mushroom types, which can be further studied in future work.

Introduction

Bacterial blotch, a complex of diseases that affects a number of mushroom species, is important to study due to its wide impact on mushroom yield and quality. Some of these diseases include, but are not limited to; Pits, Brown Blotch, Ginger Blotch, Diffuse Grey Blotch, Drippy Gill, and Marbled. There are a variety of strains that can cause these species. Some strains with recognized names, and that have been confirmed to be pathogenic are *Pseudomonas tolaassii*, *Pseudomonas agarici*, and *Pseudomonas constantinii*. On the other hand, some strains (while confirmed

pathogenic) are not recognized under official names like *Pseudomonas gingeri*, *Pseudomonas reactans* and *Pseudomonas fluorescens*[3].

It is known that at least 11 species of *Pseudomonas* cause blotch disease in Pennsylvanian mushrooms. *Pseudomonas* is the genus of a type of bacteria, it consists of multiple species that cause a variety of disease. The species are very diverse overall, with a total of 166 named species and a mere 189 remaining unnamed. The identification of *Pseudomonas* species from environmental samples had previously been accomplished using an MLSA of *gyrB*, *rpoD*, *gapA*, and *gltA* that was obtained from both named and hypothetical unnamed cliques. While there are a total of 10 recognized clades, 7 remain unnamed[3]. More work has to go into further identifying and grouping these species. By doing so, our understanding of the mechanisms of mushroom disease can be amplified.

This is particularly important for Pennsylvania, which hosts the mushroom capital of the world. Within the United States, the mushroom industry contributes an economic impact of $3.1 billion annually and creates a total of 21,000 jobs. In Pennsylvania alone, the mushroom industry induces an economic impact of $1.1 billion annually and creates more than 8,600 local jobs[1]. Essentially, this research is key to the economy.

Additionally, mushrooms have been used as medicine for thousands of years and have played an important role in oriental medicine[11]. Some commonly used mushrooms are shiitake, maitake, and reishi. Many of our recognized medicinal mushrooms have been found to have anti-tumor and immunostimulant properties, as well as being good sources for B vitamins, fiber, and antioxidants[10]. Thus, mushrooms are not only nutritious for human consumption, but provide additional benefits due to their composition. In fact, the psychedelic properties of some mushrooms could even lead to new treatments for depression and anxiety, as is being studied at John Hopkins University[6].

We want to further increase our understanding of the various pathogens causing blotch disease in order to develop management strategies, in hopes of combatting the reduction in yield and quality of our commercial mushrooms. We want to determine whether there are differences in virulence among strains on one type of mushroom and whether a given strain is more virulent on one type over another. Population dynamics is the study of how and why populations change over time, so we intend to investigate how the presence of certain pathogens has changed over time in these specific mushroom species. The goal of this research is to understand the microbial factors influencing blotch pathogens on mushroom (*Agaricus bisporus*) caps and eventually translate this knowledge to all edible mushrooms. This can lead to the development of management strategies to decrease blotch through biological control and increase commercial mushroom production.

Materials and Methods

Inoculum Preparation

The mushroom samples were treated with an inoculum of the assigned strain in media. For this project 2 [*P. gingeri]* strains and 2 strains from Clade 4 (C4) were tested; BP1480, BP1482, BP1484, BP1485. The preparation of the inoculum consisted of streaking for single colonies of the selected strains onto nutrient agar and rifampicin plates. Rifampicin is an antibiotic prescribed to treat a variety of infections. The strains were rifampicin resistant, ensuring that only these bacterial strains would grow. Single colonies of the selected strains were streaked for from a -80 °C refrigerator onto NA + Rif plates and left for 48 hours. The bacterial colonies grown were then streaked onto another plate as a lawn using a Q-tip and phosphate buffer, then left to in a 27 °C incubator for 48 hours. The bacteria were then removed from the plate used a dampened sterile cotton swab with phosphate buffer and transferred to 25 mL of 0.01M phosphate buffer. The OD was adjusted to 0.6 +/- 0.01 the day before the experiment. NA = Rif media was made for the population assay.

Collection of Mushroom Varieties

A total of 300 mushrooms were collected from Phillips Mushroom Farm in Kennett Square, PA. It was 75 of each mushroom variety (Oyster, Shiitake, Maitake, King Bolete), enough for 3 replicates per time point (T = 0, T = 0.5, T = 1.5, T = 2.5, T = 3.5). While in the growth rooms, hairnets and latex gloves were worn to avoid contamination. The mushrooms were collected in labelled brown paper bags and placed in coolers to with ice packs to keep them fresh. Once back in the laboratory setting, they were laid out in an open area until the next step.

Mushroom Preparation and Inoculation

These mushrooms were surface sterilized in bleach and rinsed with water to remove remaining bleach. The stems were cut off using a chef knife, since we only wanted to use the caps. The mushrooms were then put in a glass petri dish and assigned a treatment. They were placed randomly in plastic containers, with dampened autoclaved paper towels, to eliminate any environmental bias or impact concerns. The spread of disease can be greatly affected by environmental conditions, so a random number generator was used to assign each mushroom and treatment a number to stop this from being a concern. The mushrooms were then inoculated with their assigned treatment using a pipette, spotted with 10µL of the inoculum. The trays were then covered and allowed to incubate at room temperature for 48 hours.

Population Evaluations (Assay)

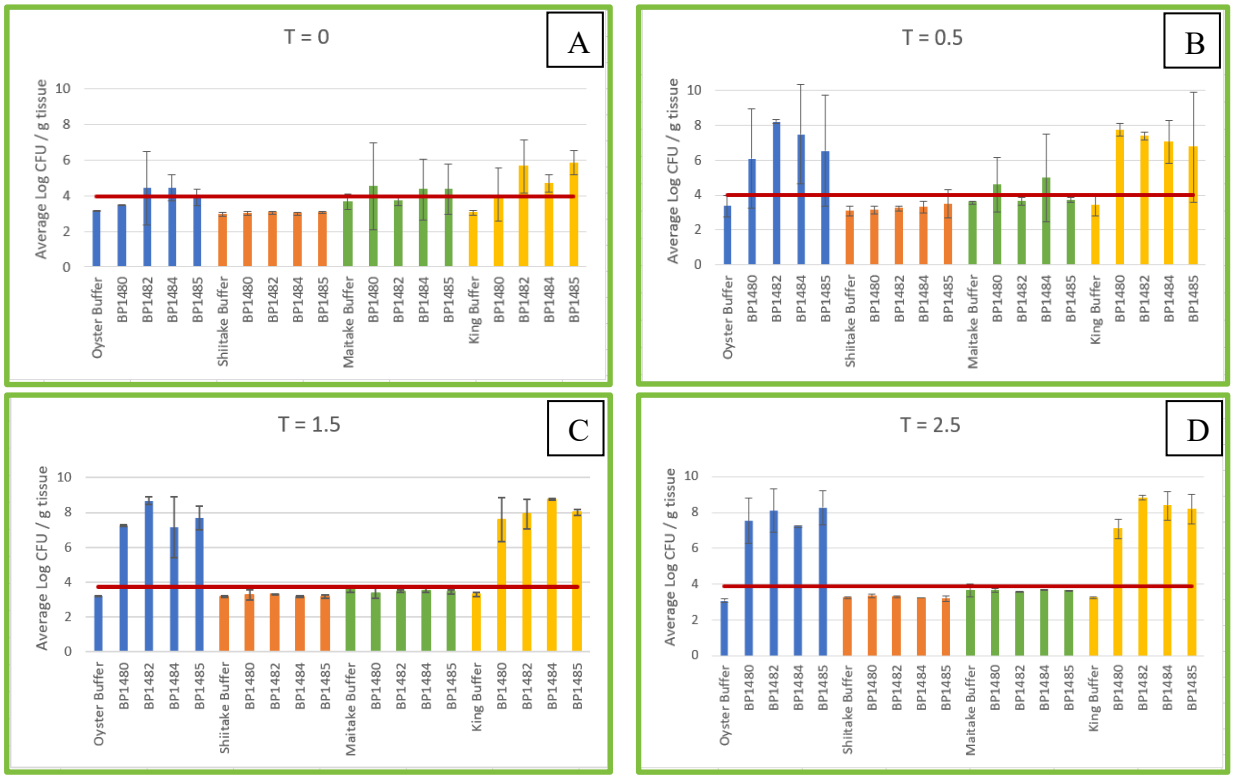
For the recovery of these samples, a cork borer and scalpel were used to remove a 1 cm^2 piece of the inoculated mushrooms. 200 mL of phosphate buffer was added to tubes and weighed, then weighed once again with the mushroom sample in. These values were subtracted to determine the weight of the sample. The mushroom pieces were then macerated with a sterile pestle and centrifuged for 10 seconds to facilitate obtaining the liquid sample from the tubes for plating. The Tissuelyser II was used to disrupt the tissue, the tubes were homogenized for 20 seconds at 20 m/s then briefly flicked. For dilution, 100 mL of the homogenate was transferred to the top row of a 96-well plate with 90 µL of 0.01 M phosphate buffer added to subsequent rows. Once the first row was filled for all columns, a multichannel pipette was used to transfer 10 µL of the first row to the second row (1:10 dilution). The samples were pipetted up and down 5 times and

then 10 µL was transferred to the next row (1:100 dilution) until 6 dilutions were done. Starting with the most dilute row, the multichannel was then used to carefully spot 5 µL 6 columns onto a labeled NA + Rif plate. After the samples were spotted onto our previously made plates and left to dry for a bit, they were left to incubate for 48 hours to allow the growth of bacterial colonies.

Data analysis

These bacterial colonies were then counted over five days to observe the disease progression, only where sufficient countable colonies were observed. These values were then log transformed to calculate the mean and variance for each strain using CFUs. The average log CFU per gram of mushroom tissue was graphed for each mushroom and treatment to be statistically analyzed, taking the limit of detection, the minimum number of bacteria that can be observed in the sample, into consideration. Observations were also recorded for any physical changes noticed in the mushrooms and pictures were taken to document this progression, utilizing a phone camera.

Results



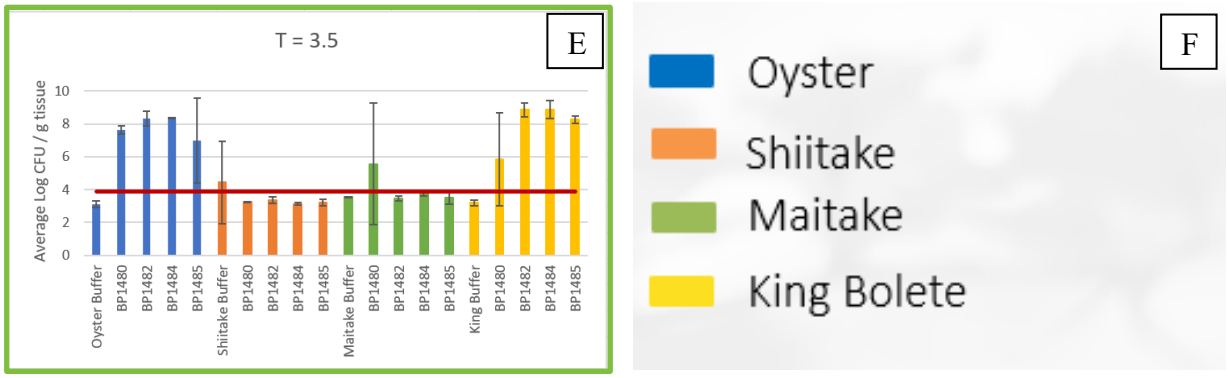


Figure 1 The average log CFU per gram of tissue was calculated for each mushroom type and treatment, then graphed per time point to observe the population dynamics per T. The red line represents limit of detection for each run. Each mushroom variety was assigned a color **(F)**. Few colonies could be observed at the time of inoculation **(A)**. However, the values increased over time for both oyster and king bolete. The values remained the same throughout for the control, the phosphate buffer, for all mushroom varieties. On the other hand, little to no colonies were observed for either maitake or shiitake mushrooms. At the end, only oyster and king bolete had significant differences **(E)**.

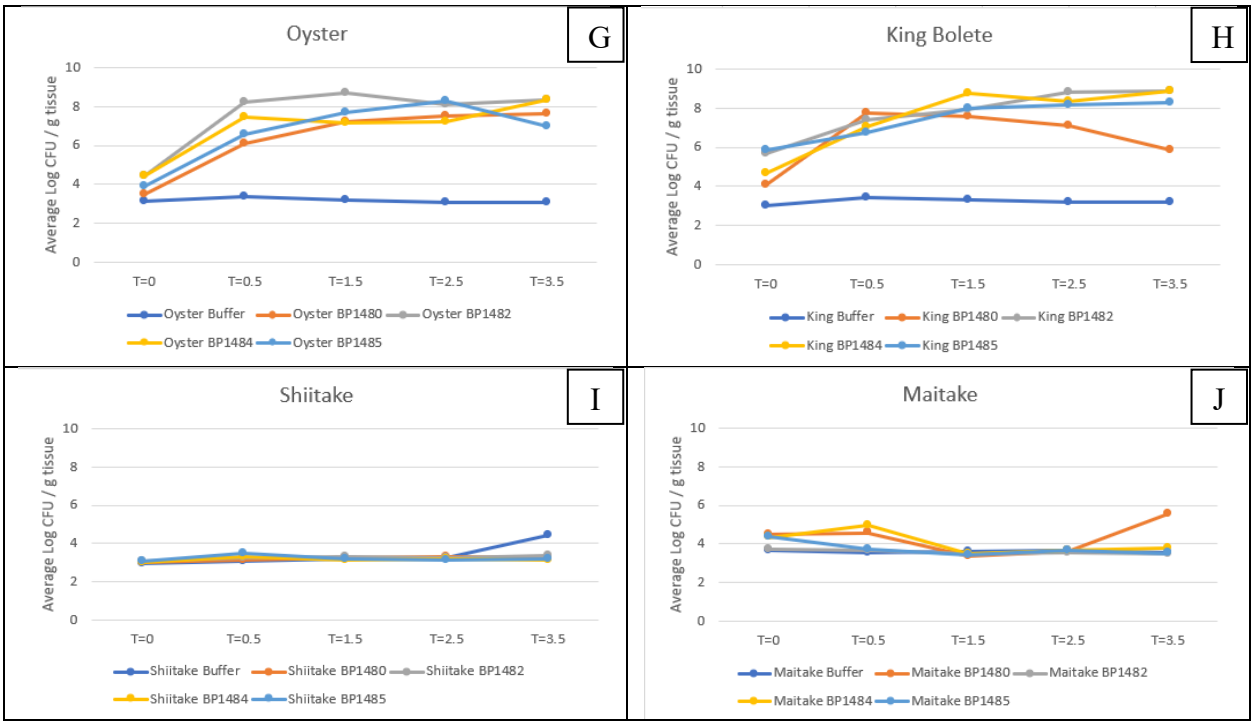
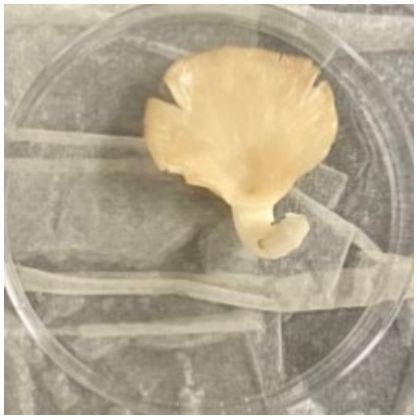
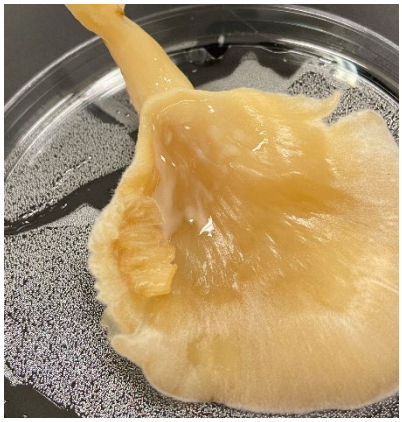


Figure 2 The results were graphed separately so we could observe the progression per mushroom variety. Both oyster **(G)** and king bolete **(H)** experienced an increase in bacterial presence over time, for all the strains. The buffer remained low for all varieties. There was minimal to no progression for both maitake **(J)** and shiitake **(I)**. Further statistical analysis has to be conducted to determine the significance of these results, specifically the trend observed for strain BP1480 in King Bolete, as it seemed to decrease slightly over time.



K

Oyster (T = 0)

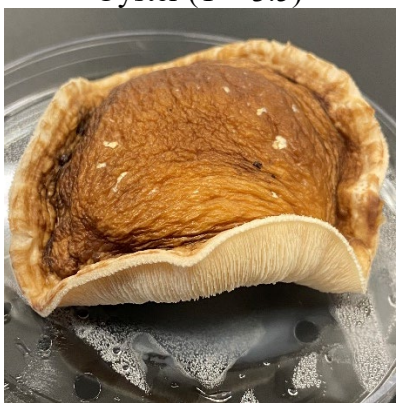


L

Oyster (T = 3.5)

M

Shiitake (T = 0)

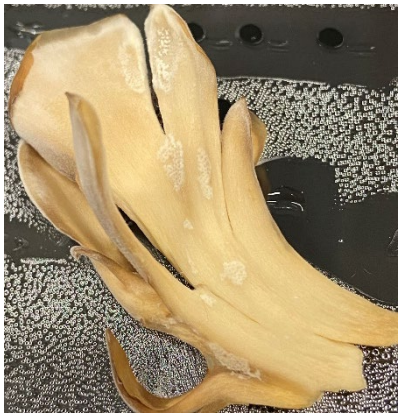


N

Shiitake (T = 3.5)

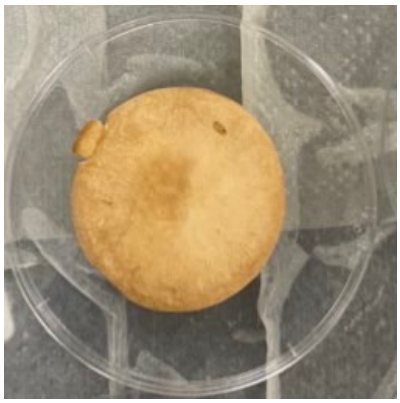
O

Maitake (T = 0)



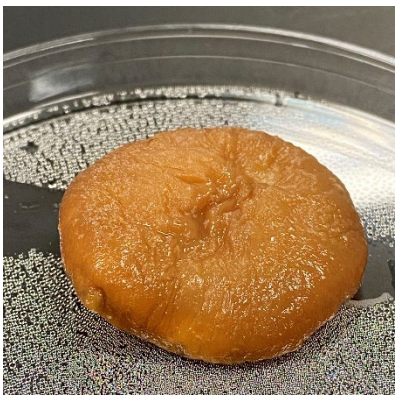
P

Maitake (T = 3.5)



Q

King Bolete (T = 0)



R

King Bolete (T = 3.5)

Figure 3 The physical differences observed were photographed. In the left column, this is what the mushrooms looked like at the beginning of the experiment (T = 0), just a few hours after inoculation. They still look relatively healthy. The right column shows how some of the mushrooms changed in appearance at the end (T = 3.5). Shiitake **(N)** and maitake **(P)** mostly showed signs of aging. On the other hand, oyster **(L)** and king bolete ® had discharge and significant change in color.

Discussion

Our results indicate that both oyster **(G)** and king bolete **(H)** mushrooms are susceptible to all the *Pseudomonas* pathogens, as they allow for the growth of bacteria in mushroom tissue. The colony forming units found per gram of tissue significantly increased over time. Thus, this is a suitable host for all the *Pseudomonas* strains they were inoculated with. On the other hand, shiitake **(I)** and maitake **(J)** mushrooms do not appear to be susceptible to these pathogens. The colony forming units per gram of tissue did not pass the limit of detection and were not physically observed on the plates after incubation. According to farm growers, blotch disease is not observed on either of these varieties, further supporting our findings.

Both shiitake and maitake are medicinal mushrooms for humans and are often found in either pill or serum form to treat a variety of health complications. Shiitake has been found to have antimicrobial properties[5]. Previously conducted studies have suggested that they have bioactive compounds that could protect against cancer and inflammation, but more work has to be conducted. Additionally, they might be able to boost immune support, improve heart health, and have promising antiviral and antibacterial properties.[7] Maitake has shown to have antibacterial, antimicrobial, and antiviral properties in previous experiments. Its components have activated several immune system cells and natural killer cells. The mushroom contains polysaccharides that that help the body's immune response and have thus been used as a booster against HIV and cancer, though more research has to be conducted.[4] Our results indicate that they have the ability to either suppress or inhibit bacterial growth of these *Pseudomonas* pathogens.

In the future, we intend to repeat this experiment again to gather more data and test for statistical significance in our findings. We want to further investigate the antibacterial and antimicrobial properties within shiitake and maitake to potentially figure out what compound(s) makes them

effective against these *Pseudomonas* pathogens. To our knowledge, no one has thought of using extracts from these mushrooms as a treatment for blotch disease in other mushrooms. If we can isolate the compound that is effective against blotch disease, a treatment could potentially be created, which would contribute to our current management strategies.

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