Interactions of Seed Bacteria with Fungal Endophytes and Plants

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D'laney C. Nimnicht

Major Professor: George Newcombe, Ph.D.

Committee Members: Stephen Cook, Ph.D.; Mark Coleman, Ph.D.

Department Administrator: Charles Goebel, Ph.D.

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Authorization to Submit Thesis

This thesis of D'laney Nimnicht, submitted for the degree of Master of Science with a Major in Natural Resources and titled "Interactions of Seed Bacteria with Fungal Endophytes and Plants," has been reviewed in final form. Permission, as indicated by the signatures and dates below, is now granted to submit final copies to the College of Graduate Studies for approval.

Major Professor: _____Date: _____
George Newcombe, Ph.D.

Committee Members: _____Date: _____
Stephen Cook, Ph.D.

_____Date:_____
Mark Coleman, Ph.D.

Department Administrator: _____Date:_____
Charles Goebel, Ph.D.

Abstract

Endophytes are microbes living symbiotically within plant tissues. They have been shown to have profound effects on plant growth and defense, yet the ecological and managerial implications of these interactions have only recently been discovered. Scientific observation and discovery in this field is sometimes difficult because interactions between the same combinations of endophytes and host plants can have radically different outcomes depending on any number of factors, both biotic and abiotic. Soil, light, temperature, genetics, and the presence or absence of other plants and microbes can all change the outcome of an interaction from mutually beneficial to neutral or even pathogenic. It's important to study these interactions further and determine how these interactions affect plant and human health. This thesis examines the effects of microbes on the growth of a common crop plant and the potential applications for human health, as well as the exclusionary interactions between endophytic bacteria and fungi present in a commercially valuable tree species with a declining population.

The first chapter reports the results of a study comparing the antagonism of microbes sourced from different plant tissues. *Pinus monticola*, commonly known as Western White Pine, was chosen the model system due to its high commercial value and declining population due to susceptibility to white pine blister rust. White pine blister rust is a fatal plant disease caused by a biotrophic stem rust, *Cronartium ribicola.* It was found that bacteria isolated from seed were more antagonistic towards endophytic fungi than those isolated from needles. These findings may be useful for future management of plant pathogens such as *Cronartium ribicola* with further testing and development.

The second chapter of this thesis describes the results of interactions between strains of *Bacillus subtilis* and *Raphanus sativus* as a model domesticated crop plant. The goal of the study was to determine if a probiotic train of human origin could have the same effects on plant emergence, growth and health as strains isolated from plant sources. The results indicate that the strains have similar affects, regardless of origin. This could have applications in agriculture as not only potential plant growth promoters, but as a novel way to deliver health promoting bacteria to human consumers.

Acknowledgements

Thank you to Dr. George Newcombe for the opportunity to work in his lab and gain valuable knowledge and experience in my chosen field. His time and patience as a mentor are appreciated. I'll always be grateful for the lessons I have learned during my time in his lab.

Thank you to Maria Marlin for her invaluable assistance in continuing her research, and her willingness to share her experience and resources whenever problems arose.

I also would like to thank Abby Ferson, Dr. Mary Ridout, and Dr. Brenda Schroeder. They have all helped me learn the practical skills needed to carry out my research, and I'll always be grateful for their guidance while I was learning.

Dedication

I would like to dedicate this thesis first and foremost to my wonderful fiancé, Josh Day. He has been incredibly supportive and caring through the entirety of this program. He is my best friend, and the one of only people who can turn tears into laughter. I cannot wait to see what life has in store for us as we move forward together.

This work is also dedicated to my father who has always been there for me and has pushed me to achieve my goals regardless of how impossible they seem. No matter how difficult things get, he has always been the rock of our family and the source of my strength, work ethic and determination. I hope I can follow your example as I build my own career going forward. Thank you again for everything!

Finally, I would like to dedicate this thesis to my late grandparents Rosemarie, and Jack. They always took a vested interest in my education and pushed me towards excellence at every stage. I know they would be incredibly proud to see how far I have come.

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Chapter 1: Comparison of Antagonism in Bacterial and Fungal Endophytes of *Pinus monticola*

Abstract

The microbiome of seeds is a highly competitive niche due to exclusionary interactions between endophytes and inhospitable conditions such as low moisture and chemical defense by the host plant. In a previous study the bacterial and fungal endophytes from *Pinus monticola* seeds were more inhibitory than fungi isolated from needle tissue. Therefore, we hypothesized that bacteria and fungi isolated from seeds would be more antagonistic than bacteria and fungi isolated from needle tissue. Two hundred and forty pairwise combinations of bacteria and fungi were tested *in agaro* to determine whether bacteria isolated from seeds of *P. monticola* were more antagonistic towards fungal endophytes than bacteria isolated from needle tissue. We found that at seven days post inoculation bacteria isolated from seed were significantly more antagonistic than bacteria isolated from needles, and that at 21 days post inoculation this difference was not significant. Fungi isolated from seeds were more inhibitory towards bacterial endophytes than fungi isolated from needles at both seven and 21 days post inoculation. These results suggest that initially, seed bacteria were more antagonistic than needle bacteria. This result could have broader implications for understanding how endophytes impact plant health and could complement efforts to manage *Cronartium ribicola,* the causal agent white pine blister rust.

Introduction

Pinus monticola, commonly known as Western white pine (WWP), once dominated Western U.S. forests from Idaho to California. This species was a source of economic strength and ecological health for the region until the late 20th century (Harvey et al., 2008). WWP populations have dramatically declined since then due several factors including fire, mountain pine beetle attack, and widespread susceptibility to an invasive plant pathogen, *Cronartium ribicola* (Dudney et al.,2020). Introduced to North America early in the 20th century, this fungus is a biotrophic stem rust that causes the fatal plant disease known as white pine blister rust. This fungal pathogen alternates primarily between white pine species and *Ribes* species such as currants and gooseberries (Fins et al., 2002; Harvey et al., 2008). Previous management efforts focused on the eradication of *Ribes* species. These efforts were ineffective due to the high prevalence of *Ribes* plants in the wild, and the presence of other more recently identified hosts for the fungus, including *Pedicularis* and *Castilleja*

species (McDonald et al., 2006). More recent efforts to reestablish this species have focused on host resistance to the pathogen and promoting the regeneration of existing natural populations. This study compliments those efforts by searching the microbiome of white pine seed and needles for endophytes that could potentially antagonize fungi that colonize needle tissue such as *C. ribicola* and enhance host resistance to pathogens. The seed microbiome is the most likely source for an antagonist to fungal pathogens due to intense genetic and enviornmental pressures causing a bottleneck of diversity and strong competition there.

This bottleneck of diversity in the seed microbiome has been documented over several decades of research and across a diversity of plant species (Mundt & Hinkle, 1976; Newcombe et al., 2018). In seeds there is most often only a singular microbe able to be cultured on potato dextrose agar (PDA). It is far more common for no PDA culturable microbes to be found at all (Newcombe et al., 2018; Raghavendra et al., 2013). In a study conducted using spotted knapweed (*Centaruea stoebe)* as the model system, it was found that seed microbes were overall less diverse and more competitive than their foliar counterparts (Raghavendra et al., 2013). Evidence from a study using a wide array of other plant hosts suggests that this may be a general pattern across many different plant and microbial taxa (Newcombe et al., 2018). Exclusionary interactions between microbes attempting to occupy the same niche, hostile abiotic conditions such as low moisture, and genetic resistance of hosts to infection are all thought to be drivers of this lack of diversity (Raghavendra et al., 2013).

Another explanation for the lack of diversity in seed colonizing microbes is found in optimal defense theory. This theory states that organisms will evolve and allocate defenses in the manner that maximizes their individual defense. In addition, this theory states that defenses are costly and take resources away from other functions, mainly growth and reproduction (Stamp, 2003). Seeds are valuable reproductive structures that require large amounts of metabolic energy to produce, therefore it is supposed that they would be strongly defended against potentially pathogenic microbes. Because defenses are costly for plants to produce, they may enter a symbiosis with plant growth promoting bacteria that have antimicrobial properties to antagonize pathogens that may infect reproductive tissues (Newcombe et al., 2018).

While endophytes isolated from seed show the most promise as a source for an antagonist to pathogens like *C. ribicola,* there are also endophytes in the needle tissue of WWP that may inhibit fungal pathogens. This study examined the pairwise interactions between 15 fungal and 16 bacterial

endophytes isolated from both *P. monticola* seeds and needle tissue and classified them into five different categories to describe the antagonistic effects of the bacteria upon the fungi. The objective of this study was to test the hypothesis that microbes isolated from seeds from WWP will demonstrate stronger antagonism than microbes isolated from needles.

Materials and Methods

Endophyte Isolation and Identification

One hundred and fifty needles from WWP saplings were collected from Idler's Rest Nature Preserve (Lat 46, Long. 116). It's owned by Palouse land trust and located approximately seven miles north of Moscow, Idaho. The needles were surface sterilized by soaking in 97% ethanol (C_2H_5OH) for one minute, 6% sodium hypochlorite (NaOCL) for five minutes, and then 97% ethanol for 30 seconds. They were air dried on paper towels in a laminar flow hood. Once sterilized, 50 needles were plated onto plates of 4% potato dextrose agar (PDA) plates and 50 were plated onto Tryptone agar. To verify the surface sterilization was successful, a random selection of sterilized needles was imprinted on PDA. All plates were incubated for 14 days at 25°C, and pure cultures of the bacterial isolates were obtained. These pure cultures were sent to Dr. Posy Busby at Oregon State University for sequencing and identification.

Experimental Design

To test if bacteria isolated from seed were more antagonistic than those isolated from needle tissue, we conducted an *in agaro* experiment using pairwise combinations of 31 endophytes from WWP. These included eight fungi isolated from seeds, seven fungi isolated from needle tissue, eight bacteria isolated from seeds, and eight bacteria isolated from needle tissue. These organisms and their GenBank accession numbers are listed in tables 1.1-1.4.

The bacteria isolated from seed and four of the bacteria from needle tissue were previously isolated by Maria Marlin in 2019 and cultures of these organisms were stored on PDA at 2.5° C. We revived these cultures by inoculating 50ml of Luria-Bertani (LB) broth with the organisms and incubating for 48 hours at 25° C. The fungal endophytes were also previously cultured by Maria Marlin on PDA, and stored at 2.5°C. These were revived by taking a 7mm plug from the edge of the colony and placing the plug in the center of a PDA plate and incubating for at least two weeks at 25°C.

Isolate label	**Species Identification**	**GenBank Accession Number**
SF1	*Penicillium sajarovii*	MK226542
SF2	*Penicillium* sp. 5 (*yarmokensearizonense* species complex)	MK226541
SF3	*Penicillium hordei*	MK226540
SF4	*Fusarium pseudocircinatum*	MK211243
SF5	*Penicillium sp. nov.*	MK226539
SF6	*Penicillium palitans*	MK410955
SF7	*Penicillium sajarovii*	MK226537
SF8	*Aspergillus proliferans*	MK211244

Table 1.1 Morphology and sequence-based identification and GenBank accession numbers for *Pinus monticola* seed fungal endophytes.

Isolate Label	**Species Identification**	**GenBank Accession Number**
NF1	*Aureobasidium pullans*	MK211236
NF2	*Elytroderma sp. nov.*	MK211237
NF3	*Coniothyrium sp.*	MK211238
NF4	*Elytroderma sp. nov.*	MK211239
NF6	*Cladosporium herbarum*	MK211240
NF7	*Alternaria sp.*	MK211241
NF8	*Lophodermium nitens*	MK211242

Table 1.2 Morphology and sequence-based identification and GenBank accession numbers for *Pinus monticola* needle fungal endophytes.

Isolate Label	Species Identification	GenBank Accession Number
SB1	*Bacillus velezensis*	MK214998
SB2	*Bacillus pumilus*	MK214999
SB3	*Bacillus velezensis*	MK215000
SB4	*Bacillus subtilis*	MK215001
SB5	*Bacillus amyloliquefaciens*	MK215002
SB6	*Bacillus amyloliquefaciens*	MK215003
SB7	*Bacillus sp.*	MK215004
SB8	*Bacillus sp.*	MK215005

Table 1.3 16s sequence-based identification and GenBank accession numbers for *Pinus monticola* seed bacterial endophytes.

Isolate	Species identification	GenBank accession number
NB 1	*Bacillus subtilis*	CP046860.1
NB 2	*Bacillus mojavensis*	MN967303.1
NB 3	*Bacillus pumilus*	CP047089.1
NB 4	*Bacillus pumilus*	CP047089.1
NB 5	*Bacillus mojavensis*	MN967303.1
NB 6	*Bacillus subtilis*	CP046860.1
NB 7	*Bacillus mojavensis*	MN967303.1
NB 8	*Bacillus aerius*	MN967235.1

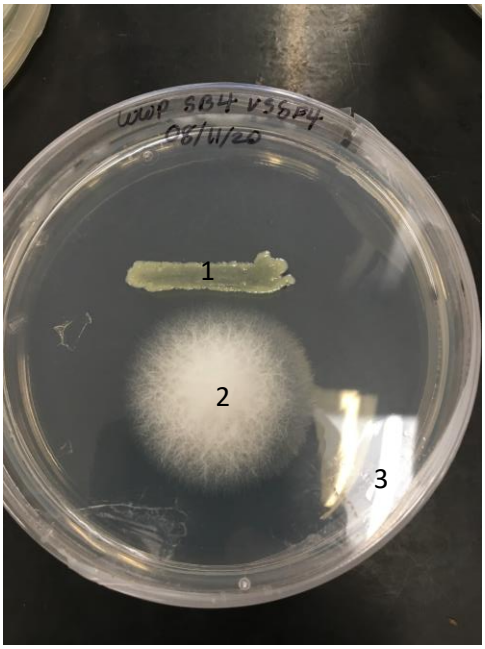
Table 1.4 16s sequence-based identification and GenBank accession numbers for *Pinus monticola* needle bacterial endophytes

Antagonism (Growth Inhibition) Assays in Agaro

Since bacterial endophytes isolated from seed displayed strong antagonistic behavior in previous studies (Marlin and Newcombe, 2019), we were interested in determining if the bacteria isolated from seeds were more antagonistic towards fungal endophytes than bacteria isolated from

needle tissue. We conducted an *in agaro* experiment with pairwise combinations of each of the 16 bacteria antagonizing each of the 15 fungi. There were three replicates of each interaction as well as control plates for a total of 813 plates.

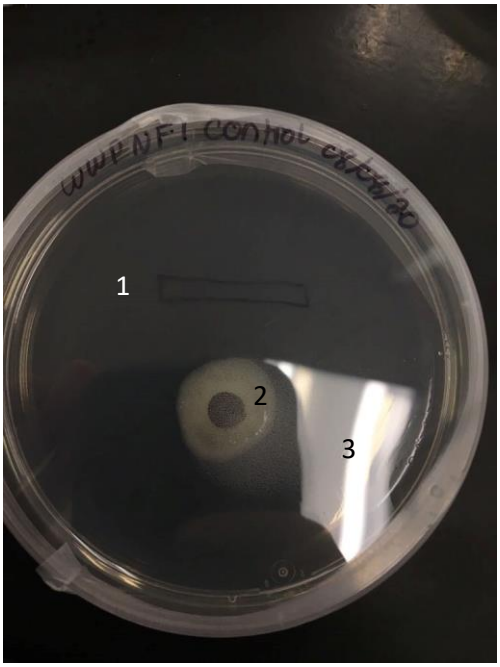
The bacterial endophytes were each grown separately in 50ml of LB broth for 48 hours. A 30 mm x 5 mm streak of bacteria was made on one side of a 60 x 15 mm plate of PDA (Figure 1.1). The fungal endophytes were grown for a minimum of two weeks, and a 7mm agar plug of actively growing fungus was taken from the growing edge of the source plate and placed 2 cm away from the bacterial streak on the assay plate, as shown in Photograph 1.1.



Photograph 1.1 Example of an assay plate used in the *in agaro* assay. 1 is the bacterial endophyte, 2 is the fungal endophyte, and 3 is reflected light on the lid of the petri dish.

Fungal Control Plates

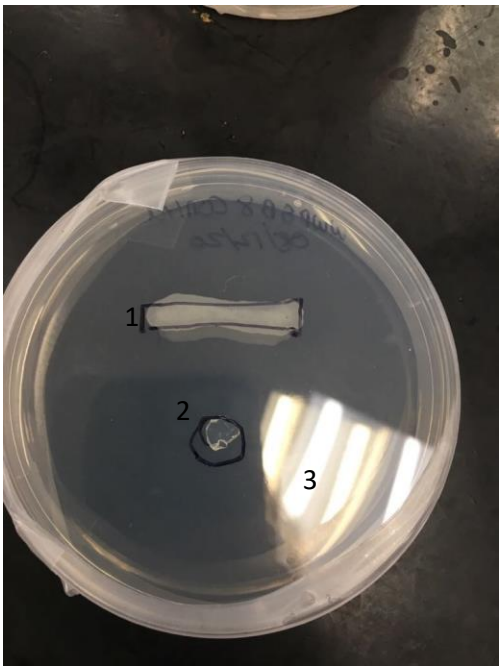
Control plates were used to observe and compare the fungal growth patterns when in pure culture to when the fungus was being antagonized by a bacterium. Colony size, growth rate and morphology were noted and compared to the characteristics of the fungi when cultured with the bacterial endophytes. A 7 mm plug of actively growing fungus was placed 2 cm away from a 30 x 5 mm streak of sterile deionized water in a 60 x 15 mm PDA plate (Photograph 1.2). The plates were repeated in triplicate for each organism.



Photograph 1.2 Example of a fungal control plate used in the *in agaro* assay. 1 is the streak of sterilized, deionized water, 2 is the fungal endophyte, and 3 is reflected light on the lid of the petri dish.

Bacterial Control Plates

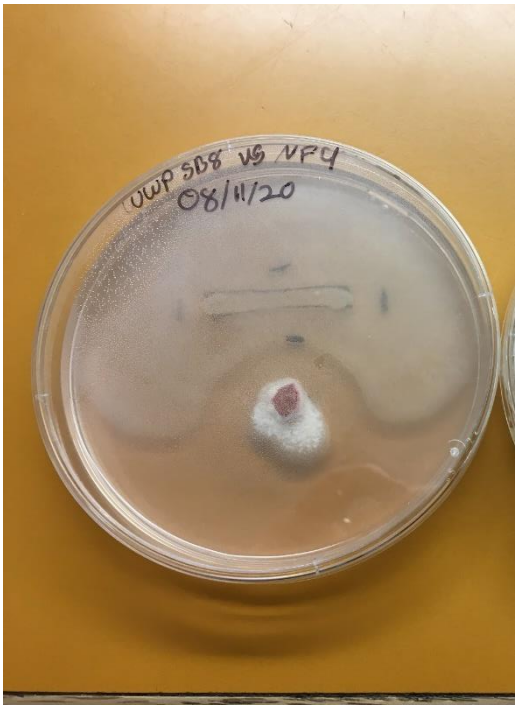
Control plates were used to observe and compare the bacterial growth patterns when in pure culture to those when the bacteria were antagonizing the fungi. These plates were used to compare the growth rate and colony characteristics of the bacteria when grown in pure culture and compared to the growth when cultured with the fungal endophytes. A single 30 x 5 mm streak of the bacteria was placed 2cm away from a 7mm plug of uncontaminated PDA on a 60 x 15 mm PDA plate (Photograph 1.3). The plates were repeated in triplicate for each organism.



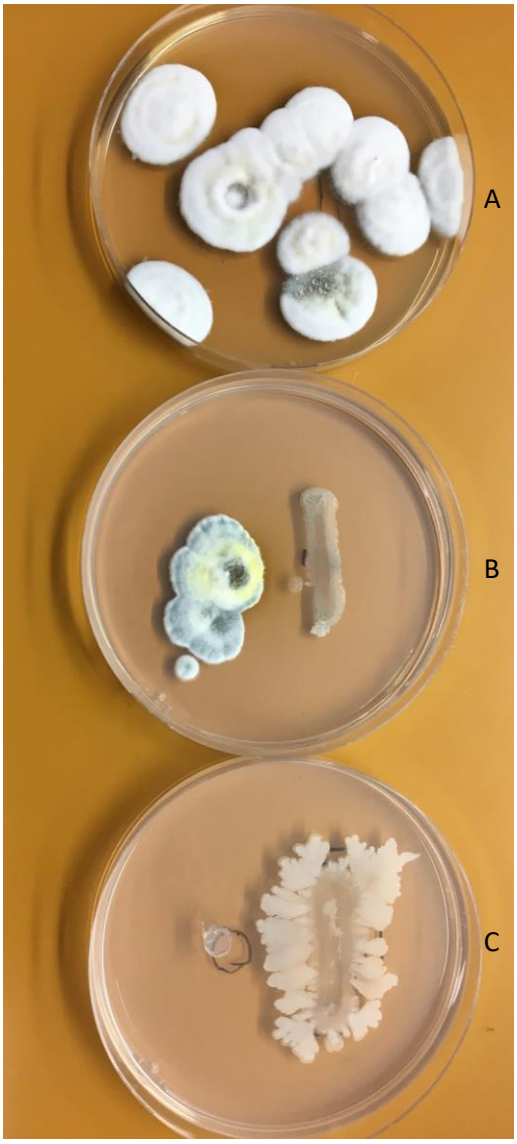
Photograph 1.3 Example of a bacterial control plate used in the *in agaro* assay. 1 is the bacterial endophyte deionized water, 2 is the plug of potato Dextrose Agar, and 3 is reflected light on the lid of the petri dish.

Data Collection

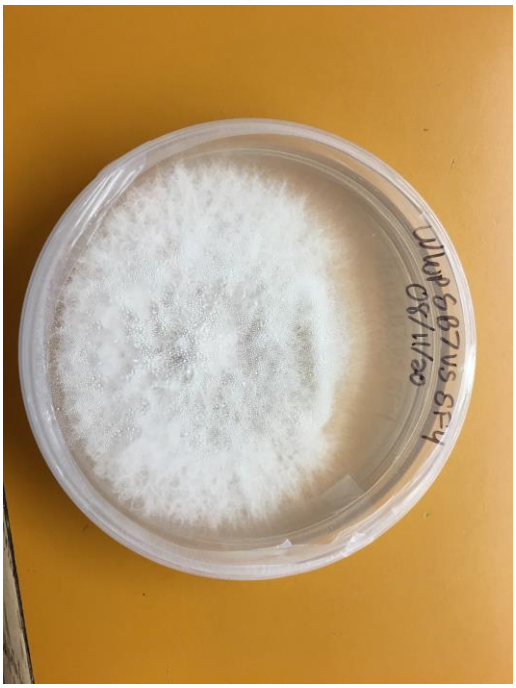
Data was taken at seven days and 21 days post inoculation. Plates were examined individually to determine if antagonism was occurring and the strength of the interaction. The interactions were scored into five categories based on the type and strength of the interaction. Category one was strong antagonism of the fungal endophyte by the bacterial endophyte (Photograph 1.4). Category three was equal antagonism or no inhibition (Photograph 1.5). Category five was strong antagonism of the bacterial endophyte by the fungal endophyte (Photograph 1.6). When the plates were contaminated or the results were inconclusive, no data was recorded.



Photograph 1.4 Example of an interaction scored as one, or strong antagonism of the fungus by the bacteria. The bacterium has nearly surrounded the fungus and has inhibited fungal growth significantly.



Photograph 1.5 Example of an interaction scored as three, which was equal antagonism. Plate A is the control plate with the fungal organism. Plate C is the control plate with the bacterial organism. Plate B shows the bacteria and fungus interacting with equal antagonism.



Photograph 1.6 Example of an interaction scored as five, which was strong antagonism of the bacteria by the fungus. The fungus has completely over run the bacterial endophyte.

Statistical Analysis

A chi-square test of independence was performed in Microsoft excel to examine the relationship between origin of the organism and the incidence of antagonism. Two tests were performed separately on the data from seven days post inoculation and 21 days post inoculation. The first test compared the antagonistic ability of bacteria isolated from seed and needles. The second test compared the antagonistic ability of fungi from seed and needles.

Results

Endophyte Isolation and Identification

Four bacterial isolates were obtained from needles and were sequenced using 16s genetic sequencing. These were labeled NB 5-8, and the species identifications and GenBank accession numbers are recorded in table 1.4. NB 1-4 were isolated in previous work done by Maria Marlin in 2019.

. *Antagonism (Growth Inhibition) Assays in Agaro*

The bacterial endophytes isolated from seed were stronger antagonists towards fungal endophytes than bacteria isolated from needle tissue at seven days post inoculation (Table 1.5). At

21 days post inoculation however, the difference was not significant (Table 1.7). The fungal isolates from seed were more antagonistic and less likely to be antagonized than needle bacteria at both seven and 21 days post inoculation (Tables 1.6,1.8). Interaction visually presenting the type and strength of each of the interactions at seven (Figure 1.1) and twenty-one days (Figure 1.2) are below. Examination of these grids allowed for visualization of the trends observed in the Chi Squared analysis.

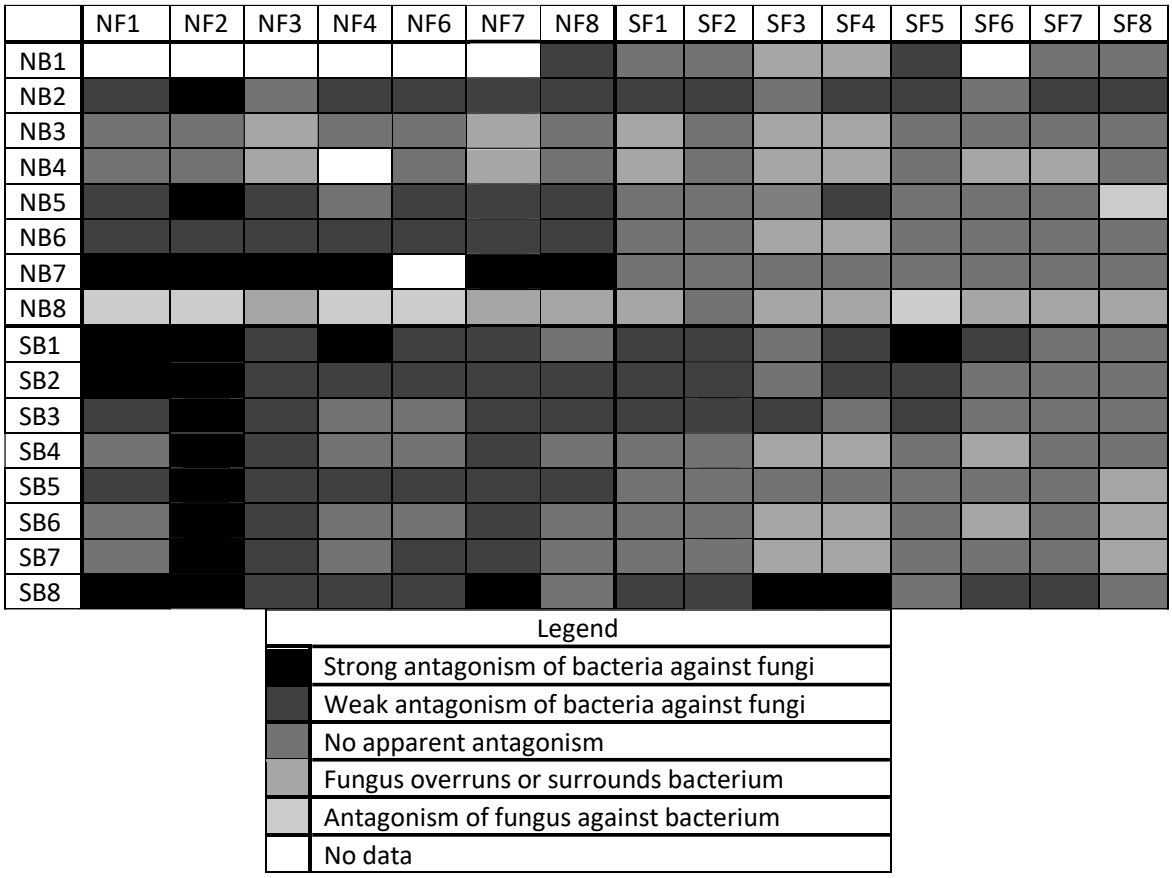


Figure 1.1 Interaction grid representing the five types of interactions observed between the bacterial and fungal endophytes. This figure visually summarizes the interactions observed at seven days post inoculation.

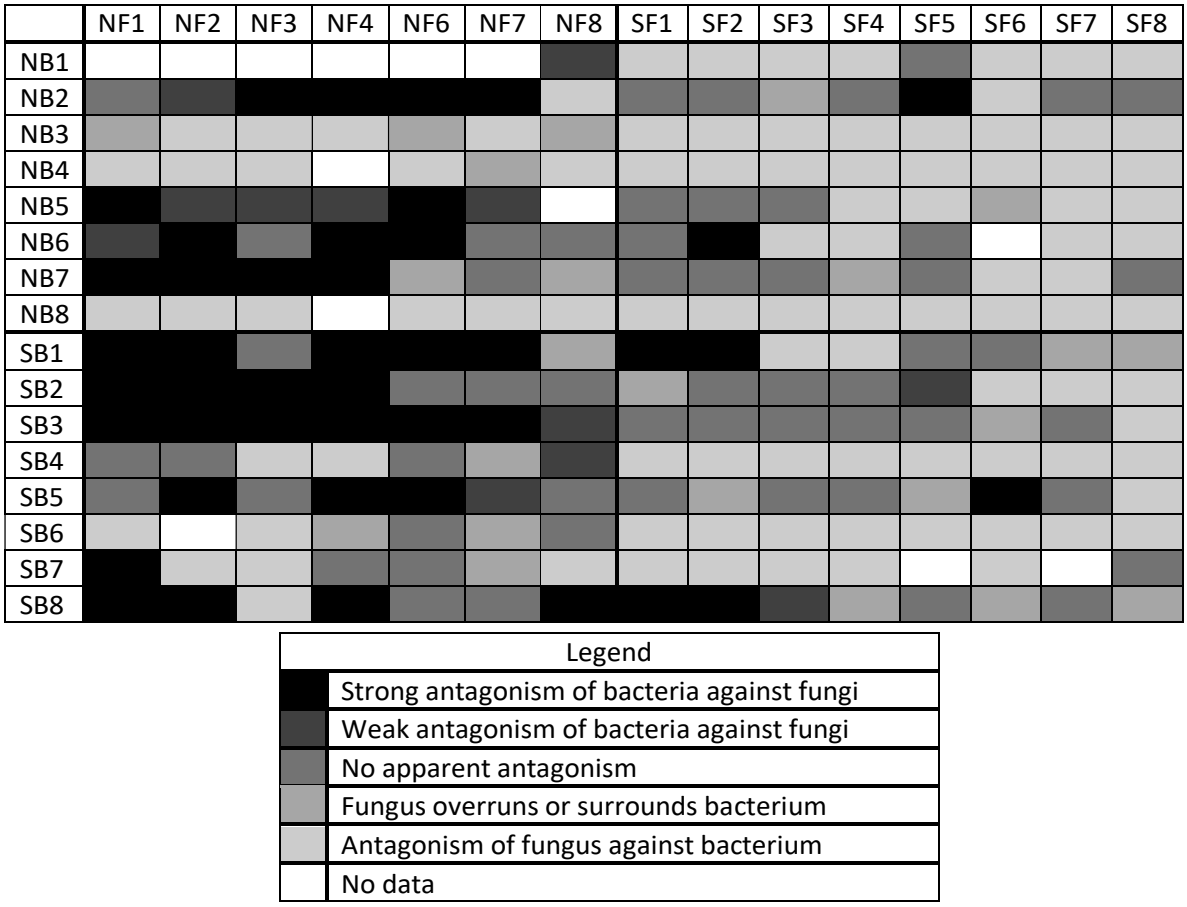


Figure 1.2 Interaction grid representing the frequencies of the five types of interactions observed between the bacterial and fungal endophytes. This figure visually summarizes the interactions observed at 21 days post inoculation.

Seven Days Post Inoculation

Bacteria isolated from seeds had equal numbers of antagonistic and non-antagonistic interactions with the fungal endophytes, with 60 instances of each type of interaction (Table 1.5). The bacteria isolated from needle tissue had more non-antagonistic interactions with the fungal isolates, with 34 instances of antagonism and 77 non-antagonistic interactions. When the Chi squared analysis was performed, the relationship between these variables was significant, $X^2(1,N=231)=8.96$, p=.0028.

Fungi isolated from seeds were more antagonistic towards bacterial isolates than fungi isolated from needles, with 100 incidences of seed fungi antagonizing bacterial isolates, and 37 incidences of needle fungi being antagonistic (Table 1.6). Fungi from needles were also more likely to be antagonized by bacteria than fungi isolated from seeds, with 67 incidences of bacteria

antagonizing fungi isolated from needles, and 27 incidences of bacteria antagonizing fungi isolated from seeds. $X^2(1,N=231)=44.13$, p=3.06E-11.

	Bacterial Antagonism against fungi	Fungal Antagonism against bacteria	Marginal row totals
Seed bacteria	60	60	120
Needle bacteria	34	77	111
Marginal column totals	94	137	231 (Grand total)
The chi-square statistic is 8.96. The *p* value is .0028.			

Table 1.5 Table summarizing chi squared analysis of data taken seven days post inoculation. The incidence of bacterial antagonism of fungi was significantly greater among the seed bacteria than the needle bacteria (Chi-square value of 8.96; p=.0028)

	Bacterial antagonism against fungi	Fungal antagonism against bacteria	Marginal row totals
Seed Fungi	27	100	127
Needle Fungi	67	37	104
Marginal column totals	94	137	231(Grand total)
The chi-square statistic is 44.13 The *p* value is 3.06E-11			

Table 1.6 Table summarizing chi squared analysis of data taken seven days post inoculation. The incidence of bacterial antagonism of fungi was significantly greater among the fungi isolated from seed than the fungi isolated from needles (Chi-square value of 44.13; p=.3.06E-11)

Twenty-one Days Post Inoculation

Bacteria isolated from seeds had far more non-antagonistic interactions, with 33 instances of antagonism and 84 non antagonistic interactions with fungal isolates(Table 1.7). The bacteria isolated from needles also had more instances of non-antagonistic interactions with the fungal isolates, with 86 non antagonistic interactions and 21 non antagonistic interactions. The relationship between these variables was not significant $X^2(1, N=224)= 2.63$, p=.1046.

Fungi isolated from seeds were more antagonistic towards bacterial isolates than fungi isolated from needles, with 115 incidences of seed fungi antagonizing bacterial isolates, and 55 incidences of needle fungi being antagonistic (Table 1.8). Fungi from needles were also more likely to be antagonized by bacteria than fungi isolated from seeds, with 45 incidences of bacteria antagonizing fungi isolated from needles, and 9 incidences of bacteria antagonizing fungi isolated from seeds. $X^2(1,N=224)= 44.64$, p=2.37E-11.

	Bacterial antagonism against fungi	Fungal antagonism against bacteria	Marginal row totals
Seed bacteria	33	84	117
Needle bacteria	21	86	107
Marginal column totals	54	170	224 (Grand total)
The chi-square statistic is 2.63. The p value is .1046.			

Table 1.7 Table summarizing chi squared analysis of data taken 21 days post inoculation. The incidence of bacterial antagonism of fungi was not significantly greater among the seed bacteria than the needle bacteria (Chi-square value of 2.63; p=.1046).

	Bacterial antagonism against fungi	Fungal antagonism against bacteria	Marginal row totals
Seed Fungi	9	115	124
Needle Fungi	45	55	100
Marginal column totals	54	170	224(Grand total)
The chi-square statistic is 44.64 The p value is 2.37E-11			

Table 1.8 Table summarizing chi squared analysis of data taken seven days post inoculation. The incidence of antagonism of bacterial isolates by fungi isolated from seed is significantly greater than fungi isolated from needles at 21 days post inoculation. (Chi-square value of 44.64; p=.2.37E-11).

Discussion

There is a considerable amount of research investigating the applications of bacterial and fungal endophytes in managing plant disease, particularly in agricultural plants such as wheat, maize, peas, solanaceous species, and ginseng and tobacco (Bevivino et al., 1998; Cho et al., 2007; Long et al., 2004; Ridout & Newcombe, 2016; Walker et al., 1998). A review of this research found that plant endophytes are most likely to be antagonistic towards plant pathogens, and that most research on the subject has focused on agricultural crop plants (Busby et al., 2016). There has been success in finding bacterial endophytes capable of suppressing multiple important crop pathogens, including several *Pythium* sp. as well as *Botrytis cinerea* and *R. solanacearum* (Compant et al., 2005; Herrera et al., 2016; Long, 2004; Rajimakers et al., 2002; Ridout & Newcombe, 2016; Walker et al., 1998; Weller, 1988). In one case, endophytes isolated from forest litter were used to control disease in wheat (Ridout and Newcombe, 2016). This suggests that forest endophytes found in a field setting might be useful in the control of other fungal pathogens.

Unfortunately, there are fewer results reported for the use of endophytes to control of plant disease in forest plants (Busby et al., 2016). However, some studies show that endophytes can

modify disease expression in forest plants as well. For example, two endophytic fungi have been found to inhibit the growth of *Endocronartium harknessii*, the causal agent of Western gall rust in hard pines (Tsuneda & Hiratsuka, 1981). Since this disease is a biotrophic stem rust, similar to white pine blister rust, it could be possible that there is an organism that could have a similar inhibitory effect on *C. ribicola.* More recently there are more promising results indicating there are endophytes from *Pinus strobus* which produce compounds with antifungal properties towards *C. ribicola* and other biologically similar organisms (Miller et al., 2012). Based on these results, bacterial endophytes could be a great asset in the management of these plant pathogens and could compliment genetic resistance found in some lineages of white pines.

Research into management of *C. ribicola* is currently focused on improving host resistance by locating the relevant genes and physiological mechanisms (Heybroek et al., 1980; Liu et al., 2013; McDonald, 1970; Keane et al., 2011). Results are promising, with findings that there are resistant lineages of *P. monticola* (Sniezko et al., 2008). Study of the mechanisms of resistance has found that there are many types of resistance and that there are many different factors that determine host resistance (King et al., 2010). The presence of bacterial endophytes has been shown to be one of these factors (Kim, 2011). Bacterial endophytes are an important part of defense against plant pathogens, and a major component of plant health for forest plants as well as agricultural plants. As such, research should include examination of the interactions and functions of bacterial endophytes in the effort to improve the resistance of *P. monticola to* white pine blister rust.

This study found that the incidence of antagonism by bacteria isolated from seed is significantly greater than bacteria isolated from needle tissue after seven days of interaction, and that there is no significant difference between antagonism after 21 days. In addition, the incidence of antagonism by fungi isolated from seeds was significantly greater than fungi isolated from needles at both seven and 21 days. These findings, along with those by Marlin and Newcombe (2019) suggest that the bacteria isolated from seed may be more antagonistic and more competitive towards fungal endophytes than bacteria isolated from needles in the short term, and that seed endophytes may be stronger antagonists. The lack of significance of bacterial antagonism at 21 days is likely due to the greater persistence of fungi. Bacteria generally have a shorter period of 24-48 hours where they are most active, where fungi will persist over a longer period. In addition, fungi isolated from seed were found to be more antagonistic and less likely to be antagonized by bacterial isolates than fungi isolated

from needles. These findings suggest that both bacterial and fungal endophytes isolated from seeds may be stronger antagonists than their foliar counterparts.

There are some limitations to these findings, especially when considering the comparison of fungal antagonism of bacterial isolates. The bacterial isolates were all *Bacillus* isolates, and the comparison of their antagonism was more direct because the organisms were coming from both the same ecological nice and taxa. The fungi were from a wide variety of taxa and this comparison is more problematic and less conclusive due to the differences between the fungal biology. In addition, quantitative data on the growth rates and size of bacterial and fungal colonies would have been useful but was not collected due to time and resource constraints, and future research could benefit from inclusion of this data.

The next step for this research is seeking replication of these interactions with endophytes isolated from other plant species. Prior research with numerous other plant species that suggests the results would be similar due to the nearly identical composition of endophyte communities across multiple plant taxa (Newcombe et al., 2018). Future findings could be applied to *in vivo* greenhouse experiments with susceptible plant hosts inoculated with *C. ribicola* to determine if bacterial endophytes from seed could improve host resistance to infection or perhaps alter disease progression in any way. This also could be tested in field experiments and applied to the management of white pine blister rust after further development. This could be commercially viable since the bacteria isolated from the plant tissue were bacillus species, which are spore forming and capable of surviving processing and transport more readily than other species. There are many possibilities for the use of endophytes in forest health and for future development.

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Chapter 2: Comparison of the Effect of Human and Plant Sourced Isolates on *Raphanus Sativus*

Abstract

Bacillus subtilis is an unusually versatile and beneficial bacterium with some variants that benefit plants while others benefit humans. Human mutualists are important members of the gut microbiome whereas plant mutualists are endophytes. Both groups show potential to diversify their communities, stimulate host defense, and improve growth and development. Ideally, human probiotic strains could be deployed in crops and benefit both crops and their human consumers. This exploratory compared a human probiotic strain of *B. subtilis, B. subtilis* 'DE111' to a mutualistic strain isolated from wheat using wheat and radish as test plants. The first experiment included a human probiotic strain of *Lactobacillus* and the following three replaced the *Lactobacillus* with a radish isolate of *Bacillus aerius*. In each of these experiments, seeds were soaked in slurries of the three bacteria and planted and emergence and dry weight were recorded. In the first two experiments, the bacteria isolated from wheat had a negative effect on biomass of radish plants. In the third experiment, *B. subtilis* 'DE111' had a positive effect on biomass of radish plants. In the fourth experiment, there was no significant difference observed. Overall, the two different sources of isolates, human and plant, had similar effects on plant growth and development.

Introduction

Bacillus species are unusually versatile and beneficial bacteria, and the range of commercial applications reflect this. *Bacillus*-based products have been developed for use not only in agricultural plants and animals but also for direct use by humans (Cutting, 2011). *Bacillus* probiotics for human consumption were first marketed sixty years ago and at least fifteen products have been developed since (Cutting, 2011). Additional products have been developed for animals in agriculture and in aquaculture and plants also benefit from inoculation with various strains of *Bacillus*. In general, strains for human use are not used for plants and vice versa, even though the application of human probiotics to crop plants could have widespread benefits for public health and agricultural productivity.

Probiotic bacteria are identified based on a wide variety of criteria, including human origin, nonpathogenic behavior, viability when being processed and transported, resistance to gastric

processes, adhesion to the outer tissue of the gut as well as antimicrobial properties and the ability to influence immune responses and metabolic activities (Duane et al., 2001). There is a rapidly growing body of literature on the possible health benefits and market opportunities for products containing these organisms. This literature has linked probiotic consumption to increased longevity, competitive exclusion of pathogens, improved mood, improvement of gastric disease symptoms, and several other benefits (Ayala et al., 2017; Benton et al., 2007; Lefevre et al., 2015; de Simone, 2019; Toohey et al., 2018; Trotter et al., 2020).

The probiotic strain of *Bacillus subtilis* known as 'DE111' belongs to *B. subtilis* subsp. *inaquosorum* (Knight et al., 2018), although that may be an unsettled designation (Harwood et al., 2018). The known benefits of 'DE111' to human health include limitation of the risk factors associated with cardiovascular disease (Trotter et al., 2020), improved body composition of female athletes (Toohey et al., 2020), and limitation of gastrointestinal distress (Cuentas et al., 2017). More broadly, the known benefits of *Bacillus subtilis* in the human gut microbiome center on the production of proteins that are instrumental for immune function (Ilinskaya et al., 2017). Presumably, these health benefits can be gained from consuming 'DE111' as well.

The agricultural sector has taken an interest in these organisms as well and *Bacillus* species have been widely used to spur plant growth, increase nutrient uptake, induce plant defense against pathogens, and lower abiotic stress (Arkhipova et al., 2005; Hashem et al., 2019; Kloepper et al., 2004: Knight et al., 2018; Mendes et al., 2013; Vardharajula et al., 2011). This is useful because there is a need for effective and environmentally friendly methods to increase yield and crop quality as well as manage pests and pathogens (Arkhipova et al., 2005; Berg, 2009; Hirt, 2020).

Several strains of *Bacillus* are already present in products used to control fungal pathogens such as *Rhizoctonia* and *Fusarium* sp. in a wide array of economically important crops such as potatoes, vegetables, ornamentals, strawberries, bulbs, turf, and wood (Berg, 2009; Compant et al., 2010). These biocontrol agents have also been used to control several different leaf spot and post-harvest diseases in crops such as sugar beet, wheat, and lettuce (Collins et al., 2003; Liu et al., 2009; Pusey, 1984; Wang et al., 2018). To our knowledge, 'DE111' has not yet been employed in plant studies, which is why it was included in this research studying the effects of bacteria isolated from humans on plant studies.

Given the potential commercial applications of these organisms and similar composition of the two microbiomes, it is surprising that there is not more literature examining the effects of human bacteria on plant growth and development, given the interest in the effects of plant bacteria on human health and agricultural productivity (Arkhipova et al., 2005; Berg et al., 2015; Hirt, 2020).

Thus, we conducted a series of four experiments that observed the effects of probiotic bacteria from both plant and human sources on radish and wheat. Our goal was to compare how the human probiotic bacteria, 'DE111', and isolates from plant sources affected plant growth. We hypothesized that the interactions would be similar due to the presence of *B. subtilis* species in both the plant and human microbiome and the easy exchange between the two microbiomes through diet (Hirt 2020).

Materials and Methods

Isolation from Probiotic Supplement

To obtain probiotic bacteria of human origin, we isolated organisms from a probiotic supplement containing active cultures. Probiotic With 'DE111' was the product purchased for this experiment. It is manufactured by Revive Herb and Deerland Enzymes based in Kennesaw, GA. We selected this product because it contained a diversity of bacterial cultures across several bacterial genera, including 'DE111'.

One pill was crushed, and the contents emptied into a sterilized beaker containing 20 ml of sterilized deionized water. To isolate the organisms contained in the probiotic capsule, approximately 2 ml of the slurry was pipetted into a sterile Eppendorf tube and vortexed to achieve homogenization. The slurry was pipetted on potato dextrose agar (PDA) and incubated at 21°C for 48 hours. After incubation, the organisms were seperated into pure culture on new PDA. One organism isolated was selected for use in the experiment and was putatively identified by Maria Marlin as *Lactobacillus rhamnosus* using Lactobacilli MRS Agar HDx from Hardy Diagnostics based in Santa Maria California.

There was a second bacterium of interest contained in this probiotic, *Bacillus subtilis* 'DE111.' This organism is known to confer various health benefits to consumers, and the effects on plant health were observed in this study. To ensure confidence in the identity of the culture, we received a known culture of this organism from Revive Herbs and Deerland Enzymes.

Isolation from Radish Seed

Bacteria were isolated from Crimson Giant radish seeds to obtain *Bacillus* isolates from a plant source to compare with the isolates of human origin. Three radish seeds were surface sterilized by first soaking seeds in 70% ethanol for 60 seconds, followed by submersion in 1ml of Tween diluted with 200 ml of sterile, deionized water for thirty seconds. Seeds were rinsed with sterile deionized water and dried with paper towels. Sodium hypochlorite (NaOCL) was not used due to concerns it would affect the seed's ability to germinate and thus release endophytes. After being sterilized, the seeds were plated on PDA and sealed with Parafilm to retain humidity. Once there was bacterial growth on the plates, pure cultures of the bacteria were obtained. One bacterium was selected for use in this project based on its' unique morphology and it was sent to Posy Busby at the University of Oregon for genetic sequencing and subsequently identified as *Bacillus aerius.* The species identification and GenBank accession number is recorded in Table 2.1.

Isolate	**Species Identification**	**GenBank Accession Number**
DE111	*Bacillus subtilis* 'DE111'	CP013984
Wheat	*Bacillus subtilis*	CP013984
Radish Isolate	*Bacillus aerius*	MN967235.1
Lactobacillus	*Lactobacillus rhamnosus*	GCA_002848015.1

Table 2.1 16s sequence-based identification and GenBank accession numbers for probiotic isolates used in these experiments.

Wheat Isolate of B. subtilis

There was a *B. subtilis* strain isolated from wheat in a previous experiment conducted by Dr. Mary Ridout at the University of Idaho. Dr. Ridout's pure cultures were stored at -2C and revived via re-culturing 5mm plugs onto PDA plates. This culture was used in our study to compare the effects of two strains of the same species of bacteria extracted from different organisms

Inoculation Experiments

Four experiments were conducted to test if human and plant sourced isolates had similar effects on plant growth. In each experiment, three separate treatments of seeds were soaked in bacterial slurries that contained one bacterium of either plant or human origin. A fourth group of seeds was soaked in sterilized, deionized water as a control. The seeds were planted in SunGro

Horticulture's professional growing mix. Emergence data was taken daily for one week before the seedlings were thinned down to one per pot, and the remaining plants were grown to maturity. The leaves and bulbs were harvested and weighed to obtain biomass. Four experiments in total were conducted due to the variability produced by context dependency in greenhouse settings. Some materials and methods were refined during subsequent experiments due to the exploratory nature of the first experiment (see Table 2.2).

Statistical Analysis

An analysis of variance and Tukey's honestly significant difference test were performed on the data to obtain P values and determine whether the differences between treatments and controls were significant. A chi squared test of independence was conducted to analyze emergence and bolting data. R Studio was used to conduct all analysis.

	Experiment 1	**Experiment 2**	**Experiment 3**	**Experiment 4**
Plant Date	02/08/19	10/30/19	03/08/20	05/31/20
Harvest Date	03/03/19	11/21/19	04/30/20	07/08/20
Number of Repetitions	10	15	15	15
Seeds per Repetition	10	3	3	3
Total Number of Radish Seeds Used	400	180	180	180
Cultivar of Radish	Crimson Giant	Crimson Giant	Crimson Giant	Crimson Giant
Wheat grown	Yes	No	No	No
Bacteria Used for Inoculation	*B. subtilis* 'DE111'	B. *subtilis* 'DE111'	*B. subtilis* 'DE111'	*B. subtilis* 'DE111
	B. subtilis isolated from wheat	B. *subtilis* isolated from wheat	*B. subtilis* isolated from wheat	*B. subtilis* isolated from wheat
	Lactobacillus rhamnosus	*B. aerius* isolated from radish	*B. aerius* isolated from radish	*B. aerius* isolated from radish
Bulbs Produced	Yes	No	Yes	Yes
Type of Weight Taken	Fresh weight of bulbs, dried weight leaves	Dry weight leaves	Dry weight bulbs and leaves	Dry weight bulbs and leaves
Fertilizer	Yes	No	Yes	Yes
Conditions	55% hum. 25°C day, 12.8°C night.	55% hum. 25°C day, 12.8°C night.	55% hum. 25°C day, 12.8°C night.	55% hum. 25°C day, 12.8°C night.

Table 2.2 A comparison of experimental methods for each of the four experiments.

Experiment One

In the first experiment, 400 Crimson Giant radish seeds were purchased from The Seed Plant, an online retailer, and seperated into four equal groups of one hundred. Each group of seeds were surface sterilized using the same procedure used in the endophyte isolation and soaked for 12h in

either a bacterial slurry, or sterilized deionized water as a control. The treatments in this experiment included *B. subtilis* 'DE111', a *B. subtilis* isolated from wheat, and the *L. rhamnosus*. isolate of human origin. Once inoculation was complete, seeds were planted into four-inch standard plastic pots. Ten seeds were planted per pot, with ten pots per treatment. This resulted in a total of 40 pots used for the experiment, with ten replicates per experimental group.

The pots were placed in a greenhouse setting with 55% relative humidity and temperatures kept at 25°C during the day, and 12.8°C at night. All ten seedlings were allowed to grow for one week, with emergence data taken daily in the morning. Once all seedlings had emerged, the seedlings were thinned to one plant per pot. Seedlings that were close to the center of the pot and of average size compared to the seedlings growing around it were selected to continue growing. The plants were fertilized once per week with Miracle-Gro fertilizer and watered daily for two weeks. At this point the radish bulbs were of marketable size and the plants were harvested.

Fresh weight of the radish bulbs was taken due to the potential relevance of fresh weight to market applications. The tops of the radishes were seperated from the bulbs and plants were individually placed in pre labeled and weighed brown paper bags. The leaves were dried at 65°C for 72 hours. Once dried, the bags containing the leaves were weighed. Biomass of the radish leaves was obtained by subtracting the mass of the empty bags from the mass of the bags containing the dried plants.

Wheat plants were also grown for this experiment and the same protocols were used for inoculating seeds, thinning seedlings, growing, and harvesting the remaining plants, and obtaining biomass through the same methods used to dry and weigh radish leaves. Wheat was included in this experiment to observe how the bacterium isolated from wheat would interact with the host plant species. Wheat was not planted or used in subsequent experiments due to limited greenhouse space available and insufficient time to grow wheat to maturity.

Experiment Two

Two hundred Crimson Giant radish seeds were purchased from The Seed Plant and were seperated into four equal groups of 45. Each group of seeds were surface sterilized and soaked for 12h in either a bacterial slurry, or sterilized deionized water. The treatments in this experiment included *B. subtilis* 'DE111', a *B. subtilis* isolated from wheat, and a *B. aerius* isolated from radish. This *B. aerius* bacterium was selected for use because we wanted to examine how an isolate from

radish plants would interact with the source plant. Once inoculation was complete, seeds were planted in four-inch standard plastic pots. Three seeds were planted per pot, with 15 pots per treatment. This resulted in a total of 60 pots used for the experiment, with 15 repetitions per experimental group.

The pots were placed in the same greenhouse conditions as in the first experiment, and the thinning, watering, and harvesting protocol was also the same. The plants that were allowed to grow were harvested and dried using the same protocol from experiment one to obtain dry weight of the leaves and this data was analyzed. The only other difference in methods was the omission of fertilizer in this experiment

Experiments Three and Four

In experiments three and four, the protocols from experiment number two were followed for inoculating seeds, thinning seedlings, growing, and harvesting the remaining plants. Two major changes were made to the methods for the final two experiments. The first was weekly fertilization with Miracle-Gro fertilizer as applied in the first experiment. The second was the drying of the radish bulbs as well as the leaves, using the same method as used in experiment two, with separate paper bags for the radishes and leaves. The final difference was that the radish plants were allowed to grow for four weeks instead of two, which allowed data to be taken on the incidence of bolting in experiment four.

Results

Overall, the effects of plant and human isolates were largely similar. Isolates from both sources had positive, negative, and neutral interactions with the plants. Some of these trends are illustrated in Figure 2.1 which compares the dry weight of leaves averaged by treatment for each experiment. This comparison was chosen because it was the most consistent across experiments due to the changes in methodologies.

The *B. subtilis* isolated from wheat had a significant negative effect on fresh weight of radish bulbs, dry weight of radish leaves and wheat emergence and in experiment one. This isolate from wheat had a significant positive effect on biomass of radish plants in the second experiment. In subsequent experiments the wheat isolate had no effect. The *L. rhamnosus* isolate from the human probiotic had a negative effect on emergence of wheat plants in experiment one, and no other

significant effects. *B. subtilis* 'DE111' had a positive effect on radish dry weight in experiment two and dry weight of radish leaves, and bulbs combined in experiment three. This bacterium had no other significant effects. The *B. aerius* isolate from radish did not have any significant effects on plant weight or emergence in any of the three experiments in which it was applied.

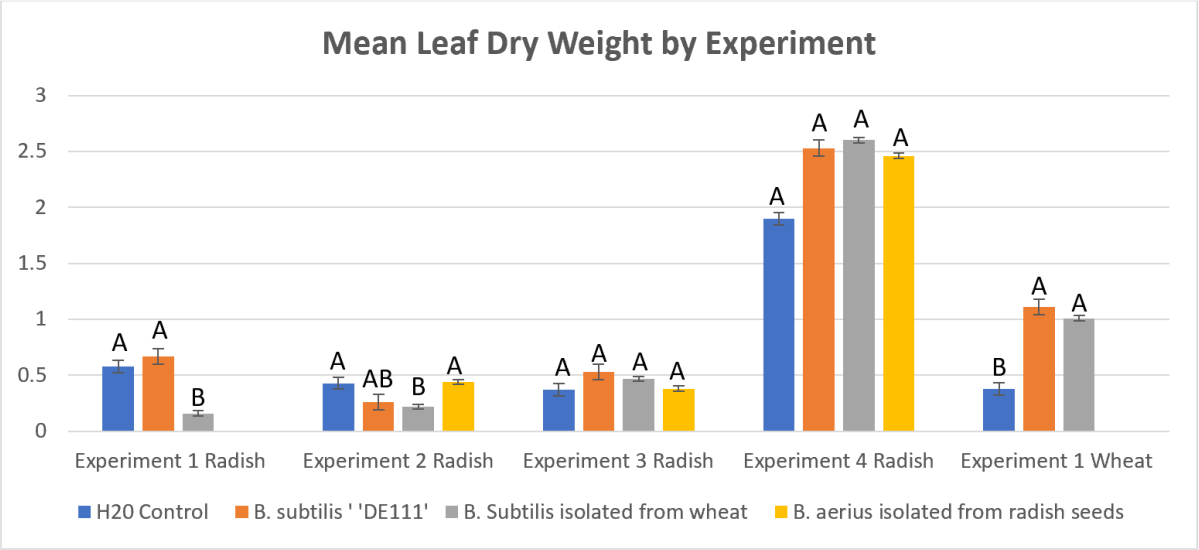

Figure 2.1 Comparison of dry weight of leaves in each experiment, averaged by treatment. Letters correspond to groupings from Tukey's honestly significant difference test. A and B are significantly different, and AB is not significantly different from either group. Standard error bars are included.

Photograph 2.1 Photograph showing noticeable size difference between treatments and control. The bulbs from the group treated with wheat is visibly smaller than the other treatments. At first examination, the bulbs treated with *B. subtilis* 'DE111' appeared larger than the control, but the difference was not significant.

Experiment One

There was a visible difference in the size and appearance of the plants in the treatments of this experiment, which was reflected in the results of the statistical analysis (Photograph 2.1).Emergence of radish seedlings was not significantly affected by any of the treatments. The *B. subtilis* isolated from wheat had a significant negative effect fresh weight of bulbs and dry weight of leaves ($p<.001$) (Figures 2.2 and 2.3). *L. rhamnosus* and *B. subtilis* **'**DE111' did not have a significant effect on the dry weight of radish leaves, or on the fresh weight of radish bulbs ($p<.05$) (Figures 2.2, 2.3).

The trends observed in radish plants were not observed in the wheat plants. The *L. rhamnosus* treatment had a significant negative affect on wheat emergence ($p<.001$) (Figure 2.4). Other treatments did not have a significant effect on emergence of wheat. Dry weight of wheat plants was positively affected by each of the three bacterial treatments ($p<.001$) (Figure 2.5).

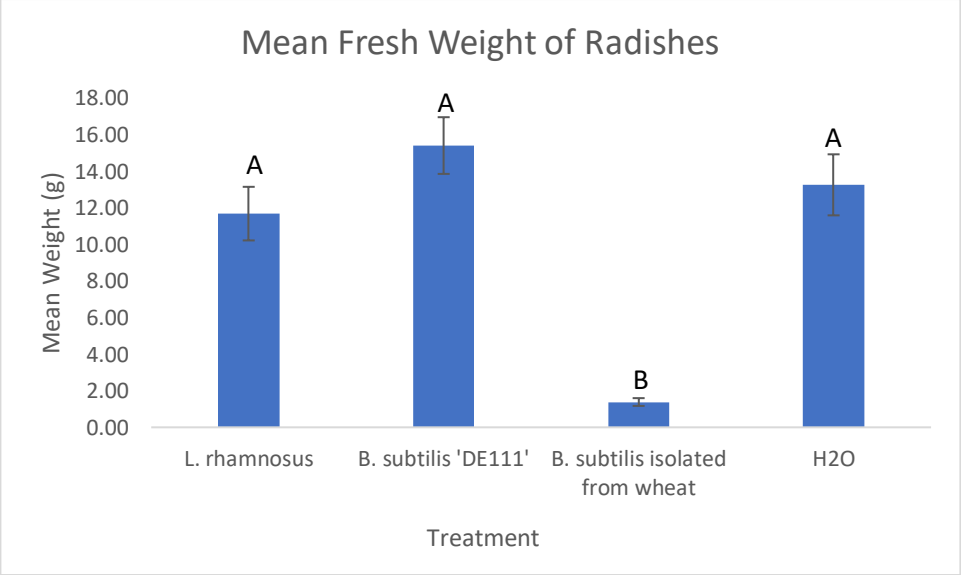

Figure 2.2 Fresh weight of radish bulbs harvested in experiment one, averaged by treatment. Letters correspond to groupings from Tukey's honestly significant difference test. A and B are significantly different Standard error bars are included.

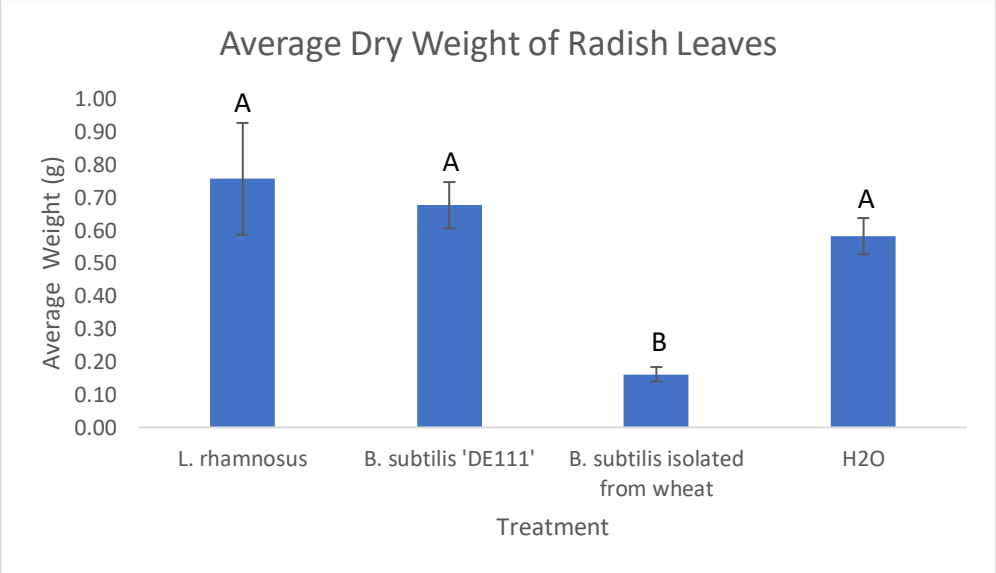

Figure 2.3 Dry weight of leaves harvested in experiment one, averaged by treatment. Letters correspond to groupings from Tukey's honestly significant difference test. A and B are significantly different. Standard error bars are included.

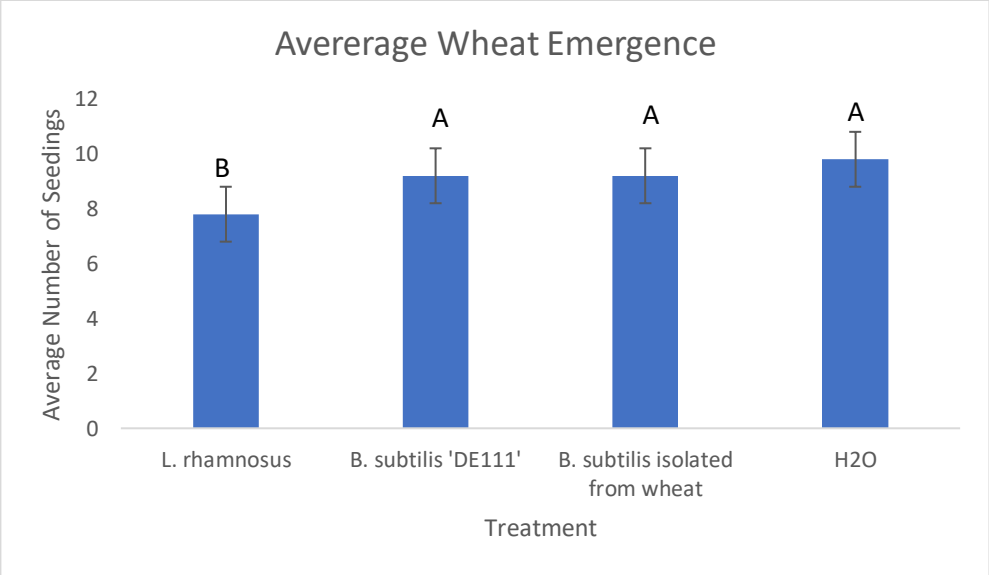

Figure 2.4 Emergence of wheat plants harvested in experiment one, averaged by treatment. Letters correspond to groupings from Tukey's honestly significant difference test. A and B are significantly different. Standard error bars are included.

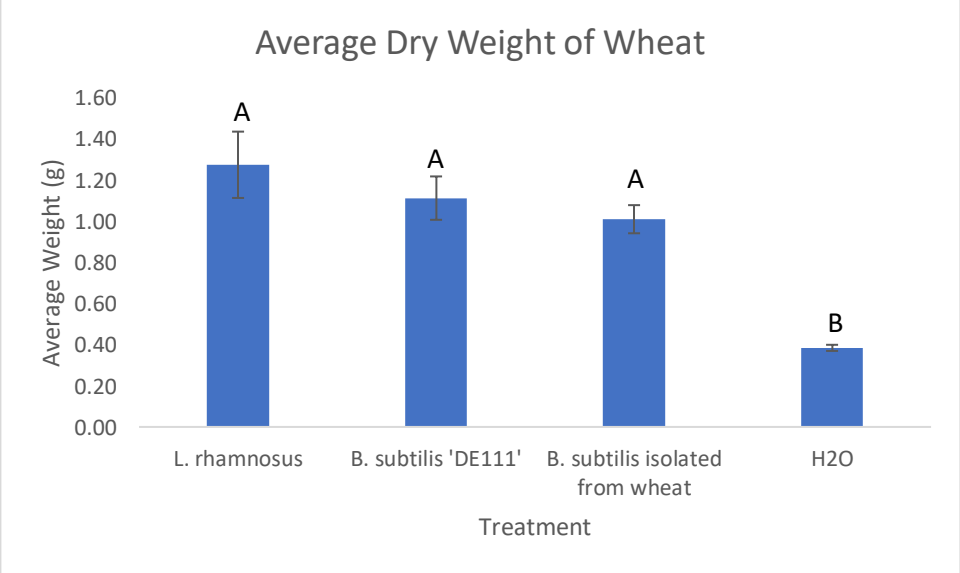

Figure 2.5 Dry weight of wheat in experiment one, averaged by treatment. Letters correspond to groupings from Tukey's honestly significant difference test. Groups A and B are significantly different. Standard error bars are included.

Experiment Two

In the second experiment, *B. subtilis* isolated from wheat had a significant positive effect on the dry weight of radish leaves ($p<.05$) (Figure 2.6). *B. subtilis* 'DE111' and the *B. aerius* from radish had no effect on dry weight of radish plants. The radish plants did not form bulbs in this experiment, most likely due to the lack of application of fertilizer.

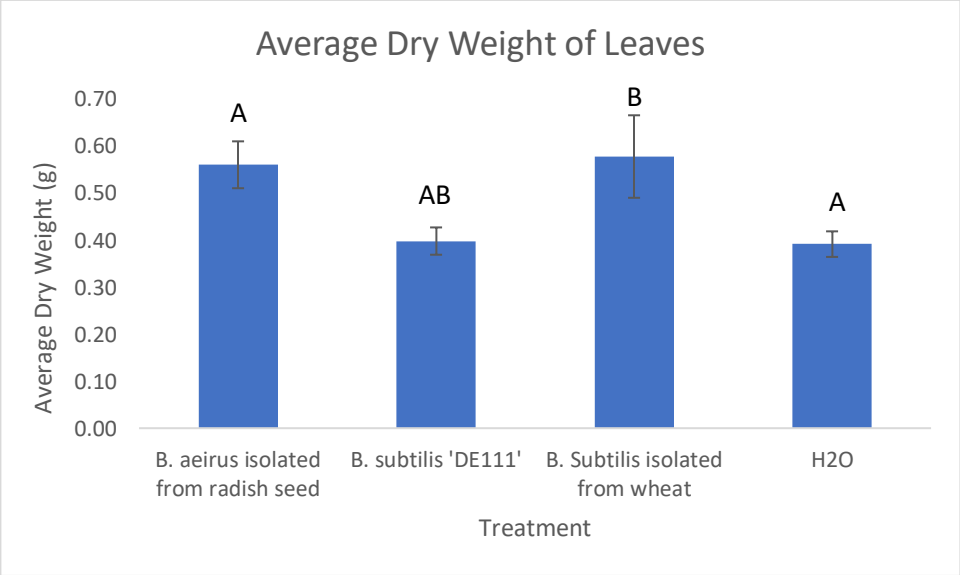

Figure 2.6 Dry weight of radish leaves in experiment two, averaged by treatment. Letters correspond to groupings from Tukey's honestly significant difference test. A and B are significantly different, and AB is not significantly different from either group. Standard error bars are included.

Experiments Three and Four

None of the bacterial treatments had a significant effect on emergence of radish plants in experiment three ($p>.05$). The treatments also had no effect on the dry weight of bulbs or leaves when analyzed individually ($p>.05$) Interestingly, *B. subtilis* 'DE111' had a significant positive effect on the combined dry weight of leaves and bulbs ($p<.05$) (Figure 2.7). In experiment four, none of the bacterial treatments had had a significant effect on emergence, dry weight of bulbs, dry weight of leaves, or incidence of bolting of the radish plants in ($p>.05$).

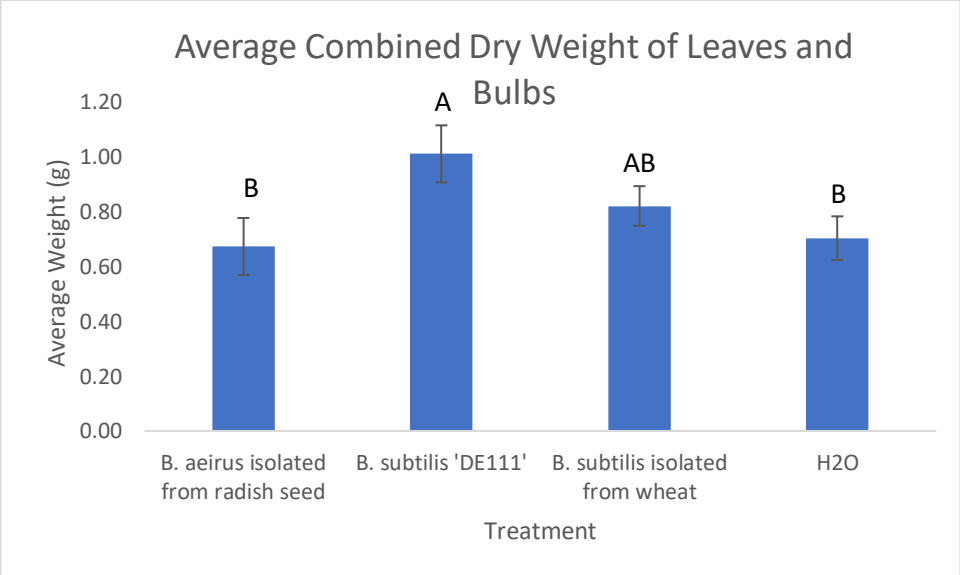

Figure 2.7 Combined dry weight of radish leaves and bulbs in experiment three, averaged by treatment. Letters correspond to groupings from Tukey's honestly significant difference test. Letters correspond to groupings from Tukey's honestly significant difference test. A and B are significantly different, and AB is not significantly different from either group. Standard error bars are included.

Discussion

This study investigated the effect of a bacterium isolated form human sources on plant growth and compared it to plant sourced isolates to observe if they would have similar effects. The human isolate, *B. subtilis* 'DE111' had both positive and negative effects on plant growth, similar to *B. subtilis* isolate from wheat. This is consistent with the literature documenting the effects of *B. subtilis* strains on plant growth, defense, and development.

This is unlike the *B. aerius* isolate from radish, which had no significant effects on plant growth in any of the three experiments where it was applied. One explanation for this could be the use of the same seeds for bacterial isolation as for the greenhouse inoculation experiments. This would mean that the endophyte would already be present, and effectively act as a control. However, this strain might be of interest in further experiments using different plant species since there is a strain of *B. aerius* which is a prime candidate for biological control against the important plant pathogen *Botrytis cinerea* due to its antifungal properties (Shafi et al., 2017). Other research suggests that different strains of *B. aerius* may also have potential as phytoremediation agents against metals like lead and chromium. (Kamaruzzaman et al., 2019; Lee & Hong, 2013). Thus, the relationship between plant growth and *B. aerius* remains unclear. Applications of this bacteria to other plant species in varied conditions may provide more conclusive results in the future.

These results showed that the human isolate and plant isolates had similar effects. This is not surprising since the two biomes have similar composition and presumably function in human and plant systems (Vilchez et al., 2016). Unfortunately, there is a tendency for interactions between bacteria and plants to change in both direction and magnitude based on biotic and abiotic factors. (Chamberlain et al., 2014). This variation is known as context dependency and studies have shown that laboratory and greenhouse settings are in fact the most variable settings for interactions and saw the most changes in interaction outcomes (Chamberlain et al., 2014).

Context dependency and the variation in the results of interactions are some of the major limitations of this study, since the strong effects observed in the first experiment were not replicated in further experiments. Other limitations of the experiment include taking the fresh weight of radish bulbs instead of dry weight in the first experiment, the failure to apply fertilizer and the omission of emergence data in the second experiment and allowing the plants in experiment four to grow for a longer period and bolt in experiment four. In addition, more measures of plant vitality could have been taken to quantify the effects of the bacteria more accurately and robustly.

More research needs to be done to determine if human isolates such as 'DE111' have potential for agricultural applications and enhance plant health. It is also possible that crops inoculated with probiotics might retain the organisms when they are harvested. If that was the case, then consumers could ingest this bacterium with their produce and obtain some health benefits from the probiotic. To determine if this is possible different varieties of crop plants would need to be inoculated with 'DE111'and tested to determine if the bacterium is still present and viable upon delivery to consumers. The next question is if the organism is present in large enough numbers to confer benefits to the consumer. The recommended number is 10^6 cfu/gram of product for probiotic organisms (Shah, 2000). If these conditions were met, these bacteria could have many unique and useful applications, and farmers could potentially work with companies like Deerland Enzymes to capitalize on growing markets for probiotic supplements and biological pest control. This may also positively affect consumer health by lowering the amount of pesticides applied to fresh foods while providing the health benefits of probiotic bacteria. There are many possibilities to be explored in this rapidly expanding field of research.

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