The Development of a Plant-Growth Promoting Biocontrol Cocktail Using Microbiological Methods and Functional Genomics to Mitigate *Salmonella enterica* Typhimurium 4/74 Contamination in Alfalfa Sprouts

A Thesis

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Dedication

This thesis is dedicated to my family: my parents, grandparents, brothers, aunts, uncles, and my fiancé. I cannot express the amount of gratitude that I have towards the support that you gave me throughout my education at the undergraduate and graduate level. Thank you!

Abstract

Salmonella enterica is a serious threat to public health that is responsible for the vast majority of reported *Salmonella* outbreaks annually. Despite interventions from private industry and governmental agencies, *Salmonella*'s main mode of transmission continues to be foodborne, thus eliminating *Salmonella* reservoirs in the food supply chain is critical to controlling this pathogen. Fresh sprouts present a unique food safety challenge due to minimal processing and ideal growth conditions created during sprouting. Currently, sprout producers have implemented interventions such as seed decontamination, regular pathogen testing of irrigation water and harvested sprouts, and post-harvest treatments. However, an intervention has yet to be introduced during the sprout germination process when conditions are the most optimal for *Salmonella* growth and proliferation. To address the current gap in pathogen reduction, great interest has been put towards the use of bacterial biocontrol agents to reduce *Salmonella* contamination during sprout germination.

In this thesis, two research goals were established. The first goal was focussed on isolating/characterizing plant-associated bacteria from alfalfa sprouts and subsequently formulating a cocktail of plant-associated bacteria that would function as a biocontrol agent to reduce *Salmonella* contamination on alfalfa sprouts. This biocontrol cocktail was then assayed for its ability to positively impact alfalfa biomass production and reduce *Salmonella* cell density on alfalfa sprouts. A plant-associated bacterial cocktail was composed of *Pantoea agglomerans*, *Priestia megaterium*, *Pseudomonas koreensis*, and *Pseudomonas putida* KT-2440. With a complex community of plant-associated bacteria, the cocktail was found to increase alfalfa biomass production by 25% when compared to uninoculated seeds while not causing a detriment to the overall shelf-life of harvested alfalfa sprouts. Furthermore, the cocktail led to a 3.1 log reduction of *Salmonella* Typhimurium 4/74 on the sprout surface after six days of germination.

The second goal was centered on utilizing a functional genomics approach through transposon insertion sequencing to determine *Salmonella* conditional gene essentiality to the colonization and infection of alfalfa sprouts. From identified conditionally essential metabolic genes, metabolites produced by alfalfa or metabolites inherent to the sprout germination environment can be targeted for consumption by biocontrol agents. Therefore, the results of the transposon insertion sequencing study could then be used to guide the process of formulating a biocontrol cocktail specifically tailored towards the consumption of metabolite targets to ultimately decrease *Salmonella* cell density on alfalfa sprouts. Due to time limitations, the results of the transposon insertion sequencing study were not available prior to the submission of this thesis for review, and the results were not included.

Table of Contents

Acknowledgements	i
Dedication	ii
Abstract	iii
Table of Contents	iv
List of Tables	viii
List of Figures	x
Chapter 1: Literature Review	1
1.1 *Salmonella*	1
1.1.1 Overview of *Salmonella*	1
1.1.2 Salmonellosis	1
1.1.3 Foodborne *Salmonella* in Food Systems	2
1.1.4 *Salmonella* Incidence in Produce and Potential Sources of Contamination	3
1.1.5 Sprouts and *Salmonella*	3
1.2 *Salmonella* Control in Alfalfa Sprout Production Systems	4
1.2.1 Current Methods	4
1.2.2 Criticisms	5
1.3 Biological Control	6
1.3.1 Overview of Biological Control	6
1.4 Microorganisms for Biological Control in Food Systems	7
1.4.1 Applications	7
1.4.2 Benefits	8
1.4.3 Deficiencies	9
1.5 Understanding *Salmonella* Growth on Alfalfa (*Medicago sativa*) Sprouts	10

Table of Contents

1.5.1 Current Knowledge of *Salmonella* Colonization and Growth on Alfalfa 10

1.5.2 Using "-Omics" Approaches to Study Genes Essential for *Salmonella* Growth on Alfalfa 11

1.5.3 Using Functional Genomics to Drive Biocontrol Agent Development 14

1.6 Conclusions 15

Chapter 2: Alfalfa (*Medicago Sativa*) Sprout Infection Model System and Biocontrol Agent Development 17

2.1 Introduction 17

2.1.1 *Salmonella*: An Overview 17

2.1.2 Impact of *Salmonella* on the General Population and the Food System in the United States 17

2.1.3 Sprouts and *Salmonella* 18

2.1.4 Current Microbial Interventions in Sprouts and Criticisms 19

2.1.5 Bicontrol as a Potential Alternative 20

2.1.6 Selection of Alfalfa as a Model System 21

2.1.7 Objectives 22

2.2 Materials and Methods 22

2.2.1 Seed Surface Decontamination and Growth Conditions for Alfalfa Sprouts 22

2.2.2 Isolation of Plant-Associated Bacteria 24

2.2.3 Characterization and Identification of Plant-Associated Bacteria 24

2.2.4 Plant-Associated Bacteria Inoculation 26

2.2.5 Biocontrol Agent Cocktail: Biomass Development and Shelf-life Study 27

Table of Contents

2.2.6 Biological Agent Cocktail: Whole Genome Sequencing 28

2.2.7 *Salmonella* Typhimurium 4/74 Inoculation 28

2.2.8 *Salmonella*-Biocontrol Agent Coinfection 29

2.3 Results & Discussion 29

2.3.1 Seed Surface Decontamination 29

2.3.2 Isolation, Characterization, and Identification of Plant-Associated Bacteria 31

2.3.3 Plant-Associated Bacteria Inoculation 41

2.3.4 Biocontrol Agent Cocktail: Biomass Development, Shelf-life Study, and Whole Genome Sequencing 43

2.3.5 *Salmonella* Typhimurium 4/74 Inoculation 54

2.3.6 *Salmonella*-Biocontrol Agent Coinfection 55

2.4 Conclusions & Future Directions 57

Chapter 3: Characterization of Essential Genes to the Infection and Colonization of Alfalfa by *Salmonella* Typhimurium 4/74 Through Transposon Insertion Sequencing 59

3.1 Introduction 59

3.1.1 Current Knowledge on *Salmonella* Genes Essential for Alfalfa Infection and Colonization 59

3.1.2 Using Functional Genomics to Drive Biocontrol Agent Development 59

3.1.3 The Basis of Transposon Insertion Sequencing, Potential Deficiencies, and its Application in Studying *Salmonella* Gene Essentiality *in vitro, in vivo,* and *in planta* 60

3.1.4 Objectives 62

3.2 Materials & Methods 63

3.2.1 Development of Transposon Mutant Library 63

Table of Contents

3.2.2 Inoculation of Alfalfa with Transposon Mutant Sublibraries 64

3.2.3 Aggregation of Sublibraries and Preparation of Cells for gDNA Extraction 65

3.2.4 Preparation of Samples for TnSeq Analysis: Methodology #1 66

3.2.5 Preparation of Samples for TnSeq Analysis: Methodology #2 71

3.2.6 Data Analysis of TnSeq Results 71

3.3 Results & Discussion 71

3.3.1 Development of Transposon Mutant Library 71

3.3.2 Inoculation of Alfalfa with Transposon Mutant Sublibraries 73

3.3.3 Data Analysis of TnSeq Study 75

3.4 Conclusions & Future Directions 79

Chapter 4: Conclusion 81

Bibliography 83

List of Tables

Table 1: Primers used to amplify the 16s rRNA gene sequence in the PAB isolates. 25

Table 2: PCR reaction preparation components to amplify the 16S rRNA gene sequence in the PAB isolates. 25

Table 3: Conditions for each PCR reaction to amplify the 16S rRNA gene sequence in the PAB isolates. 25

Table 4: Constituents of the plant-associated bacterial biocontrol cocktail and source of isolate. 27

Table 5: The qualitative characteristics of the plant-associated bacterial isolates regarding colony morphology on LB, Gram stain, cell morphology, and ability to grow on XLT4 agar. 33

Table 6: The identification of 16 plant-associated bacterial isolates extracted from three different alfalfa sources. 39

Table 7: Qualitative observations regarding the shelf life of surface decontaminated Food to Live alfalfa seeds that have been either treated with a biocontrol agent cocktail or left untreated. 46

Table 8: The BV-BRC genome ID associated with each PAB cocktail constituent in addition to the number of contigs that resulted from genome assembly, the estimated genome length, the GC content, and the number of open reading frames (ORFs). 48

Table 9: The genes comprising a type VI secretion system and the copy number of each gene identified in *Pantoea agglomerans* JV6. 52

Table 10: The genes comprising a type VI secretion system and the copy number of each gene identified in *Pantoea agglomerans* RG18. 52

Table 11: Primers used for linear extension, dsDNA synthesis, and amplification of transposon specific sequences to prepare a Tn-Seq amplicon library. 67

Table 12: Linear extension reaction preparation components and the conditions for each linear extension reaction to create linear extension products from the 5’ end of the Tn5 transposon. 68

Table 13: A-tailing reaction components used to A-tail linear extension products. 68

Table 14: dsDNA synthesis reaction preparation components and the reaction conditions programmed to the thermocycler. 69

Table 15: Thermocycler conditions used to amplify the Tn-Seq amplicon. 70

List of Tables

Table 16: The recorded time constant and resulting transformation efficiency for each transformation experiment performed. The number of *Salmonella* Tn5 mutants allocated to each sublibrary. 72

Table 17: The concentration of ssDNA and dsDNA that was measured throughout the TnSeq library preparation workflow prior to Illumina sequencing. The negative control consists of day 6 sheared gDNA without the Taq 2x Master Mix added to the reaction during the initial linear extension step. 75

List of Figures

Figure 1: Photographs of the large-scale (A) and small-scale (B) environments used to achieve sprouting conditions. 23

Figure 2: Percent germination and percent contamination of either ISS or FTL alfalfa seeds based on a varying percentage of commercial bleach used in the seed decontamination process. 30

Figure 3: The cell density of plant-associated bacteria extracted and enumerated from treated and untreated International Specialty Supply alfalfa seeds over a three day period of time 31

Figure 4: The cell density of plant-associated bacteria extracted and enumerated from treated and untreated Food to Live alfalfa seeds over a six day period of time. 32

Figure 5: The development of alfalfa biomass in response to a 4.5 to 5 log CFU/g inoculation of plant-associated bacteria and subsequent incubation for a 6 day period of time. 42

Figure 6: The development of plant-associated bacterial cell density *in planta* following a 3 to 5 log CFU/g inoculation and subsequent incubation for a 6 day period of time. 42

Figure 7: The development of alfalfa biomass in response to a 7 log CFU/g inoculation of a plant-associated bacteria cocktail and subsequent incubation for a 6 day period of time. 44

Figure 8: The development of plant-associated bacterial cell density *in planta* following a 7 log CFU/g inoculation and subsequent incubation for a 6 day period of time. 45

Figure 9: A photograph of an LB plate that indicates each PAB cocktail constituent and reflects the abundance of each PAB cocktail constituent extracted from alfalfa sprouts following six days of germination. 45

Figure 10: A phylogenetic tree comparing homology in the sequences of 100 single-copy genes of JV6 to genomes of *Pantoea spp.* available in the BV-BRC database. 49

Figure 11: A phylogenetic tree comparing homology in the sequences of 100 single-copy genes of JV24 to genomes of *Priestia spp.* available in the BV-BRC database. 49

Figure 12: A phylogenetic tree comparing homology in the sequences of 100 single-copy genes of RG1 to genomes of *Pseudomonas spp.* available in the BV-BRC database. 50

Figure 13: A phylogenetic tree comparing homology in the sequences of 100 single-copy genes of RG18 to genomes of *Pantoea spp.* available in the BV-BRC database. 50

List of Figures

Figure 14: The cell density of *Salmonella* Typhimurium 4/74 extracted and enumerated from treated and untreated Food to Live alfalfa seeds grown on water-agar over a six day period of time. 54

Figure 15: The cell density of *Salmonella* Typhimurium 4/74 extracted and enumerated from Food to Live alfalfa seeds that were inoculated with either the PAB cocktail & *Salmonella*, *Salmonella* only, or a PAB cocktail constituent & *Salmonella*. 55

Figure 16: A general workflow of the protocol adapted from Karash et al. used to create a Tn-Seq amplicon library for Illumina sequencing. 66

Figure 17: The cell density of seven sublibraries of Tn5 mutant *Salmonella* Typhimurium 4/74 cells extracted and enumerated from surface decontaminated Food to Live alfalfa seeds grown on water-agar over a six day period of time. 74

Figure 18: A TBE gel image displaying TnSeq amplicons of the 0, 1, 3, and 6 day samples. 77

Figure 19: The coverage of the day 0 amplicon sequences that align with a reference *Salmonella* Typhimurium 4/74 genome, and the GC content of each aligned sequence. 78

Chapter 1: Literature Review

1.1 *Salmonella*

1.1.1 Overview of *Salmonella*

Salmonella is a genus of Gram-negative, rod-shaped, and facultatively anaerobic bacteria belonging to the family Enterobacteriaceae (Eng et al., 2015). Within this genus, there are two species: *S. bongori* and *S. enterica* that have been differentiated based on differences in their 16S rRNA genomic sequence (Brenner et al., 2000; Eng et al., 2015). *S. enterica* has been further classified based on genomic relatedness and biochemical properties into six distinct subspecies: *enterica, salamae, arizonae, diarizonae, houtenae,* and *indica* (Brenner et al., 2000; Eng et al., 2015). Furthermore, the World Health Organization has identified 2,579 serotypes of *Salmonella spp.* using the Kauffmann-White scheme (Grimont & Weill, 2007). The Kauffmann-White scheme differentiates serotypes of *Salmonella spp.* based on differences in the reactivity of *Salmonella* surface antigens such as the somatic (O) antigen and flagellar (H) antigen to anti-serums (Diep et al., 2019). The species/subspecies with the most prolific number of serotypes belongs to *S. enterica* subsp. *enterica* with 1,531 serotypes of the 2,579 known serotypes of *Salmonella spp.* (Grimont & Weill, 2007). Additionally, *S. enterica* subsp. *enterica* serotypes have been attributed to causing 99% of *Salmonella*-related infections in humans and warm-blooded animals (Jajere, 2019). However, a select number of serotypes of *S. enterica* subsp. *enterica* are responsible for the majority of *Salmonella*-related infections. According to Jackson et al., a Center for Disease Control and Prevention (CDC) study in 2009 discovered that only 20 serotypes of *S. enterica* subsp. *enterica* were responsible for over 82 percent of the approximately 36,000 *Salmonella* isolates collected from reported human *Salmonella*-related infections in that year (Jackson et al., 2013). For brevity henceforth, *S. enterica* subsp. *enterica* will be referred to as *Salmonella*.

1.1.2 Salmonellosis

Salmonellosis is a gastrointestinal infection with key symptoms of diarrhea (with or without blood), fever, and stomach cramps; however, other symptoms such as nausea and vomiting, may be observed (CDC: Symptoms, 2019). Cases of salmonellosis present within 8 to 72 hours post-infection, and the infection persists for several days for the average person (Mayo Clinic: Salmonella Infection, 2019). On a global scale, *Salmonella* is estimated to be responsible for 93.8 million illnesses and 155,000 deaths annually (Majowicz et al., 2010). In the United States (US), *Salmonella* is responsible for an estimated 1.2 million illnesses each year (Jackson et al., 2013). Although cases of salmonellosis vary in severity depending on the specific serotype of *Salmonella* and the health of the infected person; children under the age of 5, the elderly, and immunocompromised people are highly susceptible to *Salmonella* infections compared to the average healthy person (Eng et al., 2015). Therefore, the aforementioned populations would be more susceptible to developing complications such as severe dehydration,

bacteremia (presence of bacteria in the bloodstream), and reactive arthritis (Mayo Clinic, 2019). Salmonellosis normally passes without the need for additional treatments except for the intake of fluids and electrolytes (CDC: Diagnosis & Treatment, 2019). However, in severely complicated cases, antibiotics are commonly administered to clear the bacteria (Mayo Clinic, 2019). The use of antibiotics in the treatment of salmonellosis has given rise to several antibiotic resistant strains of *Salmonella* that are impervious to antibiotics such as fluoroquinolones and 3rd generation cephalosporins (Jarere, 2019). Therefore, a greater emphasis must be placed on finding and eliminating the reservoirs of *Salmonella,* which will ultimately prevent severely complicated cases of antibiotic-resistant salmonellosis in susceptible populations. *Salmonella* is ubiquitous in the environment and natural reservoirs such as poultry, cattle, swine, and wild birds have been documented (Jarere, 2019). However, of the 93.8 million reported cases of *Salmonella* globally, it is estimated that 80.3 million cases are attributed to foodborne outbreaks (Majowicz et al., 2010). Therefore, reducing the prevalence of *Salmonella* in the food supply chain would eliminate a key reservoir that is known to contribute towards human disease.

1.1.3 Foodborne Salmonella in Food Systems

In the US, greater than 75% of *Salmonella* illnesses were attributed to the following seven categories: chicken, fruits, pork, seeded vegetables, other produce such as nuts, turkey, and eggs (Interagency Food Safety Analytics Collaboration, 2021). Furthermore, products such as beef, vegetable row crops, sprouts, dairy, and fish contributed to the remaining 25% of attributed *Salmonella* illnesses (Interagency Food Safety Analytics Collaboration, 2021). Of the 1,531 serotypes of *Salmonella*, four particular serotypes have been identified as key epidemiologically important serotypes in foodborne illness: *Salmonella* Typhimurium, Enteritidis, Heidelberg, and Newport (Jarere, 2019). These four serotypes were responsible for ~350 outbreaks between 2007 and 2011 in the US; however, these serotypes remain largely restricted to certain food categories (Andino & Hanning, 2015). *Salmonella* Enteritidis is commonly associated with animal products such as eggs, chicken, pork, and beef. *Salmonella* Typhimurium is associated with chicken, leafy greens, and peanut butter. *Salmonella* Heidelberg is associated with chicken, turkey, and dairy products. *Salmonella* Newport is associated with sprouts, vegetables, tomatoes, pork, and poultry (Andino & Hanning, 2015). Despite enacted regulations in the US, the relative rate of annual *Salmonella* infections remained the same from 2008 to 2014 (Crim et al., 2015). However, the incidence of *Salmonella* Typhimurium decreased while the incidence of other serotypes such as *Salmonella* Javiana and *Salmonella* Infantis increased (Crim et al., 2015). Therefore, the wide diversity of *Salmonella* leads to an ebb and flow of serotypes throughout time without changing the overall incidence of *Salmonella* infections. Thus, the US continues to be afflicted with *Salmonella* leading to an economic burden estimated at $4.14 billion as of 2020 (USDA & ERS, 2020).

1.1.4 Salmonella Incidence in Produce and Potential Sources of Contamination

Based on data collected in 2019, approximately 41.8 percent of *Salmonella*-related foodborne illnesses were attributed to fruits, seeded vegetables, vegetable row crops, sprouts, and other produce (Interagency Food Safety Analytics Collaboration, 2021). Within a five year period (2017-2021), fifteen of the thirty-nine multistate outbreaks of *Salmonella* related to food have been reported in fresh produce (CDC: Reports, 2022). Furthermore, three particular outbreaks (2019-2021) that were attributed to contaminated peaches, onions, and cut melons resulted in 1,278 reported illnesses and 326 hospitalizations (CDC: Melons, 2019; CDC: Onions, 2022; CDC: Peaches, 2020). Therefore, *Salmonella* contamination in produce continues to have a significant impact on consumers in the US.

Salmonella is an enteric species that inhabits the gut of its host, and *Salmonella* serotypes' main mode of transmission is via the fecal-oral route (Gopinath et al., 2012). Therefore, the main source of contamination in the food system is by fecal contamination caused by the shedding of *Salmonella* from infected wild animals, domestic animals, and humans (Hilbert et al., 2012). However, *Salmonella* is well-known as an environmental pathogen that can persist in soil, water, or food processing facilities for extended periods of time, thus the elimination of contamination sources or harborage points is a difficult task (Carrasco et al., 2012; Strawn et al., 2013). According to Carrasco et al., surface water provides an ideal harborage for *Salmonella* especially when the surface water is located next to a pasture containing domesticated animals or the surface water attracts wild animals that can shed *Salmonella* into the water supply (Strawn et al., 2013). Furthermore, the *Salmonella*-laden water has the potential to contaminate soil where *Salmonella* has been found to persist in soils that are moist, have poor drainage, and has close proximity to a surface water source (Strawn et al., 2013). As a result, soil and irrigation water are two pre-harvest sources of *Salmonella* contamination that pose a significant risk to produce contamination during its cultivation (Fatica & Schneider, 2011; Harris et al., 2003). Sources of *Salmonella* contamination in produce have also been linked to post-harvest processing facilities. In these facilities, one source of contamination is the washing step where produce is washed to remove *Salmonella* persisting on the surface of the fruit or vegetable; however, this wash water can become contaminated thus creating a harborage for *Salmonella* (Carrasco et al., 2012). Furthermore, *Salmonella* has been found to persist on surfaces such as conveyor belts, stainless steel, polyvinyl chloride (PVC) tubes, and wood surfaces for more than 21 days after processing (Fatica & Schneider, 2011). Therefore in the produce processing system, *Salmonella* has the potential to occupy numerous pre-harvest and post-harvest harborage points that can act as a potential source of contamination.

1.1.5 Sprouts and *Salmonella*

Within the produce category, sprouts were attributed with causing 4.2 percent of *Salmonella*-related foodborne illnesses (Interagency Food Safety Analytics Collaboration,

2021). Sprouts have progressively gained a greater consumer interest in Western cultures since the 1980's due to a higher demand for less-processed produce with fewer additives that can be used as garnishes on salads, sandwiches, or other high-quality foods (Benincasa et al., 2019). Furthermore, sprouting is an inexpensive process that occupies a minimal amount of greenhouse space, has a short growth cycle to reach harvest maturity, requires minimal processing equipment, and results in relatively high yields of sprouts (Kyriacou et al., 2016; Lorenz & D'Appolonia, 2009). According to the National Advisory Committee on Microbiological Criteria for Foods (NACMCF), sprouts are grown in a dark, humid environment for an average of 3 to 7 days at 21 to 26°C, and the sprouting environment varies from plastic 5-gallon pails, 32-gallon barrels, sprouting beds, or growth chambers optimized for sprout production (NACMCF, 1999). However, sprouting conditions such as time, temperature, water activity, pH, and available nutrients have been found to promote the growth of foodborne pathogens such as *Salmonella* (FDA, 2017). As a result, there have been twenty-two *Salmonella*-related outbreaks between 1999 and 2020 recorded in the United States that have resulted in 1003 reported illnesses (Miyahira & Antunes, 2021). Furthermore, the contamination of sprouts resulted in a high-profile case where the Food and Drug Administration (FDA) filed a warning letter to the US-based sandwich chain Jimmy John's due to five independent outbreaks of *Salmonella* and *Escherichia coli* that were associated with sprouts served at several Jimmy John's locations (FDA, 2020). The warning letter stated that Jimmy John's had not taken significant corrective measures to prevent the sale of adulterated sprouts in their stores despite repeated interventions over an eight year period of time (FDA, 2020). Therefore, sprouts present a rapidly expanding market with increased consumer interest that is highly susceptible to contamination by foodborne pathogens such as *Salmonella* that has led to numerous multistate outbreaks of Salmonellosis.

1.2 *Salmonella* Control in Alfalfa Sprout Production Systems

1.2.1 Current Methods

To address the incidence of foodborne pathogens in produce, the FDA implemented a rule, within the scope of the Food Safety Modernization Act (FSMA), named the Standards for Growing, Harvesting, Packing, and Holding of Produce for Human Consumption (Produce Rule) (Produce Rule, 2015). Under this ruling, the FDA implemented the following five requirements for sprouting facilities (Produce Rule, 2015):

1. Establish scope of applicability of sprout provisions.
2. Establish measures that must be taken related to seeds or beans for sprouting.
3. Establish measures that must be taken for growing, harvesting, packing, and holding sprouts.
4. Require testing the growing environment for *Listeria* species or *Listeria monocytogenes* and testing each production batch of spent sprout irrigation water

or sprouts for *Escherichia coli* O157:H7, *Salmonella* species, and under certain conditions, other pathogen(s), and taking appropriate follow-up actions.

5. Require certain records, including documentation of treatment of seeds or beans for sprouting, a written environmental monitoring plan, and sampling plan, test results, certain test methods used, and corrective actions.

According to the NACMCF, sources of contamination of sprouts can be traced to improperly treated water, animal waste, poor sanitation of equipment, and/or poor personal hygiene of workers; however, sprouting seeds are likely to be the main source of contamination (NACMCF, 1999). Chemical treatments using compounds such as chlorine, acetic acid, or calcium hypochlorite are typically used to decontaminate the surface of seeds resulting in an average reduction of 2 logs (Mir et al., 2021). However, research has shown that methods such as acidic electrolyzed water, cold plasma, high pressure processing, hot water, ultrasound, pulsed light, ultraviolet irradiation, and gamma irradiation have also been used to decontaminate the seed surface (Mir et al., 2021; Puligundla et al., 2017; Wuytack et al., 2003). The efficacy of these various treatments varies quite dramatically with hot water and gamma irradiation achieving 6 to 7 log reductions, respectively, whereas ultraviolet irradiation was found to only achieve a ~1 log reduction (Mir et al., 2021).

Another intervention occurs after the sprouts have been harvested. Chemical treatments, acidic electrolyzed water, cold plasma, gamma irradiation, and ultraviolet irradiation have been studied as potential post-harvest intervention methods (Mir et al., 2021). Similar to seed decontamination, chemical treatments resulted in a 2 log reduction of the background microflora on the treated sprouts (Mir et al., 2021). Oh et al. reported that the use of Nitrogen cold plasma resulted in a 2.6 log reduction of *Salmonella* Typhimurium on the surface of radish sprouts (Oh et al., 2017). Finally, 2 kGray of gamma irradiation was reported to cause a 5 log reduction of *Salmonella* in alfalfa, mung bean, and radish sprouts (Rajkowski & Thayer, 2000).

1.2.2 Criticisms

Although there are a plethora of methods that have been developed to control *Salmonella* in sprouts, each method may not be effective or practical due to each technology's own limitations. In terms of chemical rinses and ultraviolet irradiation being used to decontaminate the surface of seeds, these interventions lead to a 1 to 2 log reduction in *Salmonella*; however, the NACMCF recommends a 5 log reduction to effectively eliminate *Salmonella* from the seed surface (Mir et al., 2021; NACMCF, 1999). Furthermore, the use of chlorine compounds such as chlorine dioxide and hypochlorous acid can degrade into chloroform, chlorophenol, and trihalomethane, known carcinogens, that can remain on either the seed surface or harvested sprouts as residues (Mir et al., 2021). Additionally, methods such as electrolyzed oxidized water, ultraviolet light, cold plasma, and chemical washes are limited to the surface of a treated product (Mukhopadhyay &

Ramaswamy, 2012). These surface level treatments present a severe limitation because *Salmonella* is known to internalize inside of the sprout tissue via two methods: chemotaxis into the stomata of the plant tissue when in the presence of light and colonization of lateral root cracks (Dong et al., 2003; Gandhi et al., 2001; Golberg et al., 2011). As a result, internalized *Salmonella* would be impervious to the aforementioned surface-level treatments. An additional challenge that continues to hinder the implementation of control methods such as high pressure processing is the scalability of this process to a continuous industrial manufacturing environment (Mukhopadhyay & Ramaswamy, 2012). Although gamma irradiation showed promising results in reducing *Salmonella* by 5 to 7 logs in finished sprouts and seeds, respectively, irradiation of foods continues to have an unfavorable public perception (Mir et al., 2021; Shen et al., 2017). Based upon these criticisms, there is a high demand for continued innovation of current control methods and the discovery of novel control methods of *Salmonella* in sprouts is imperative.

1.3 Biological Control

1.3.1 Overview of Biological Control

Biological control or biocontrol is defined as the introduction of a natural or genetically modified organism to a system to manage the population of undesirable and/or harmful pests (Thakur & Singh, 2018). According to Eilenberg et al., there are four types of biocontrol: conservation, classical, inoculation, and inundation biocontrol (Eilenberg et al., 2001). Classical biocontrol is described as the introduction of an exotic biocontrol agent that will become established and serve as a form of long-term pest control (Eilenberg et al., 2001). Inoculation biocontrol is the process of intentionally releasing a biocontrol agent into a system knowing that the agent will multiply in number; however, the biological control agent's presence will not be permanent (Eilenberg et al., 2001). Inundation biocontrol is the intentional release of a biocontrol agent to control pests; however, the initial population of biocontrol agents are incapable of reproduction due to factors such as dispersal or inactivation (Eilenberg et al., 2001). Therefore, in contrast to inoculation biocontrol, the success of inundation biocontrol lies entirely on the initial population of biocontrol agents and not their progeny. Conservation biocontrol is the process of modifying the environment or changing existing conservation strategies to protect and enhance natural enemies of a pest to reduce the pest population (Eilenberg et al., 2001).

A substantial branch of biological control constitutes the use of microorganisms such as bacteria, fungi, viruses, and protozoa (Harding & Raizada, 2015; Kevan & Shipp, 2017). These microbial agents are often used as biopesticides or bioherbicides. For example, the bacterium *Pseudomonas fluorescens* WH6 has been found to produce an extracellular metabolite labeled Germination Arrest Factor that inhibited the germination of 29 different plant species including common nuisance grass species (Banowetz et al., 2008). Microbial biocontrol agents have also been utilized in the prevention of plant pathogens. Rhizosphere bacterial species such as *Bacillus*, *Agrobacterium*, *Pseudomonas*, and *Erwinia* have been found to produce antibiotic compounds such as iturin A, surfactin,

agrocin 84, phenazine derivatives, pyoluteorin, pyrrolnitrin, and herbicolin A that have the potential to control plant pathogenic bacterial species (Ab Rahman et al., 2018). Additionally, Molinari and Leonetti have shown that tomato seedlings inoculated with a fungal-bacterial community lead to a significant increase in the transcription of salicylic acid-dependent pathogenesis-related (PR) genes which forms a plant's systemic acquired resistance (SAR) (Molinari & Leonetti, 2019). This increased transcription of PR genes subsequently led to a stronger expression of chitinase and glucanase genes in the tomato plant because these enzymes are used as a defense mechanism against root-knot nematode infections (Molinari & Leonetti, 2019). The induction of plant SAR mechanisms to elicit the production of peroxidase and chitinase to counter a root-knot nematode infection was also observed in cucumber plants that was initiated by the fungal biocontrol agent *Trichoderma harzianum* (Yedidia et al., 1999).

1.4 Microorganisms for Biocontrol in Food Systems

1.4.1 Applications

As previously discussed, microorganisms can play a significant role in the food supply chain as bioherbicides and biopesticides in agriculture. However, biocontrol can be applied further in the food supply chain where microbial biocontrol agents can be utilized as biopreservatives against toxigenic microorganisms or foodborne pathogens.

One application of biocontrol in the food supply chain is the use of *Aspergillus flavus* AF36, an atoxigenic strain of *Aspergillus*, to reduce the presence of toxigenic strains of *Aspergillus flavus* on cotton, maize, and ground nuts (Moral et al., 2020). The contamination of aflatoxin poses a serious threat to the health of humans and livestock animals because chronic exposure to aflatoxins can lead to symptoms such as reduced growth rate, immunosuppression, liver cancer, and/or decreased food conversion (Moral et al., 2020). However, the application of *Aspergillus flavus* AF36 and similar strains of atoxigenic *Aspergillus flavus* has shown a 70% to 100% decrease in toxigenic strains of *Aspergillus flavus* on field crops such as cotton, maize, and ground nuts (Moral et al., 2020).

Another antimicrobial biocontrol agent that is heavily utilized in the food industry are bacteriophages. Bacteriophages are viruses that infect and replicate in host bacterial cells, then the host bacterial cell is lysed to release newly replicated phage (Kasman & Porter, 2021). Therefore, bacteriophages make ideal candidates for the biopreservation of food. Bacteriophage have been found to cause reductions ranging from 1 to 6.8 log against pathogens such as *Listeria monocytogenes, Salmonella,* and *Campylobacter* in products such as meat, poultry, dairy, fruit, sprouts, leafy greens, and other vegetables (Hertwig et al., 2013). In the case of controlling *Salmonella*, companies have commercialized preparations of lytic bacteriophage under the trade names SalmoFresh™, SalmoLyse©, and PhageGuard™ that have been found to reduce *Salmonella* on raw chicken products 2.5 logs, 0.9 logs, and 2 logs, respectively (Hertwig et al., 2013).

The application of bacteria to food for biopreservation is another major strategy of biocontrol and this practice has been used since ancient times to preserve various types of foods (Gálvez et al., 2010). Lactic acid bacterial species such as *Lactococcus lactis, Enterococcus faecium, Lactobacillus plantarum, Lactobacillus sakei,* and *Lactobacillus curvatus* have been used for centuries in the biopreservation of fermented meat and dairy products (Arqués et al., 2015; Castellano et al., 2017). These various bacterial species produce antimicrobial peptides, bacteriocins, that have been found effective against *L. monocytogenes* and *Salmonella* in fermented meat products (Castellano et al., 2017). Furthermore, lactic acid bacteria mainly preserve fermented food products through the production of lactic acid to lower the food's pH to 5.4 to 3.22 (Beganović et al., 2011; de Souza et al., 2019; Vignolo et al., 2010). Through this acidification, the growth of foodborne pathogens such as *L. monocytogenes* and *Salmonella* is inhibited when the pH of a food is lowered below 4.4 and 3.7, respectively (FDA, 2016). However, bacterial biopreservation is not solely limited to lactic acid bacteria. The application of plant-associated bacterial species such as *Pseudomonas, Pantoea, Serratia, Enterobacter,* and *Hafnia* have been found to cause a 1 to 5 log reduction of *Salmonella* in alfalfa sprouts and fresh cut lettuce leaves (Fett, 2006; Oliveira et al., 2015). However, *Pseudomonas* displayed the most promising effect on *Salmonella* with a 3 and 5 log reduction in lettuce leaves and alfalfa sprouts, respectively (Fett, 2006; Oliveira et al., 2015).

1.4.2 Benefits

In terms of bacterial biopreservation, numerous plant-associated bacterial biocontrol agents are also considered plant-growth promoting bacteria because these species positively impact the growth of the host plant through the production of growth stimulating hormones, fixation of nitrogen, and the facilitation of nutrient acquisition via organic acids or siderophores (Morales-Cedeno et al., 2021). Therefore, these plant-growth promoting bacteria serve a dual purpose of stimulating plant growth while also inhibiting the growth of plant pathogens pre-harvest and inhibiting the growth of human foodborne pathogens & spoilage organisms post-harvest (Hsu & Micallef, 2017; Markland et al., 2015; Morales-Cedeno et al., 2021; Wang et al., 2021). As of 2007, the New Zealand Institute of Food Science & Technology acknowledged that the use of naturally occurring bacterial biocontrol agents has the greatest acceptance by consumers (McIntyre et al., 2007). Additionally, a consumer-perception study found that consumers displayed a greater interest in more natural preservation methods such as biopreservation in fruits compared to the current chemical treatments (Mesías et al., 2021). Furthermore, biopreservation species such as lactic acid bacteria are probiotics meaning that some of these species can colonize/inhabit the large intestine and confer a myriad of benefits such as the production of nutrients and enhancement of their absorption; the promotion of good digestion; and the stimulation of the adaptive immune system (Kuar et al., 2015). Furthermore, once established in the gut, lactic acid bacteria can have an antagonistic effect towards pathogens through the following mechanisms: competitive exclusion for space on the surface of the

gut's epithelial cells, the production of antimicrobial bacteriocins, the consumption of limiting nutrients, and the stimulation of the host's immune system to increase IgA secretion into the gut cavity (Hossain et al., 2017).

In comparison to non-thermal and thermal treatments that are applied to foods, biocontrol offers an additional benefit in that the organoleptic and sensory properties of the treated food may be retained because the bacterial biocontrol agents are endemic to the food product such as is the case with lactic acid bacteria in fermented foods and agricultural crops associated with an endemic plant-growth promoting bacteria (Gálvez et al., 2010). In comparison to treatments such as irradiation or chemical washes, biopreserved food products can be marketed as a "clean label" product i.e. the food has not been heavily processed using traditional reduction methods (Perpetuini et al., 2021). Clean label is defined broadly by Asioli et al. as a food product that meets the following criteria: the package has certification logos; the front of the package is simple; and the packaging has statements such as 'free-from preservatives/additives', organic, and natural (Asioli et al., 2017). Since the 1980's, consumer demand has increased rapidly for a wider variety of clean label products, and this demand has been equally matched in the development of clean label products by the food industry to fill this growing market (Asioli et al., 2017). Therefore, biocontrol agents such as lactic acid bacteria or plant-associated bacteria have the potential to be marketed as natural, biopreservation/biocontrol agents that may also provide probiotic effects towards the consumer depending upon the biocontrol agent utilized.

In relation to the cost of producing bacterial biocontrol agents, waste by-products generated by the food industry such as molasses, peanut hulls, corn cobs, fish meal, chitin sources, yeast extracts, and soybean hulls can be utilized as carbon and nitrogen sources to produce relatively low cost biocontrol agent formulations at a large scale (Fravel et al., 1999; Yánez-Mendizábal et al., 2012). As an example, the production cost of a biocontrol agent formulation composed of *Pseudomonas fluorescens* and *Pseudomonas chlororaphis* was modeled to cost \$20.51 and \$3.31 per liter of forumulated biocontrol agent at a small and large scale production environment, respectively (Olanya et al., 2016).

1.4.3 Deficiencies

Although biocontrol can appear to be an excellent alternative to traditional methods to control against pathogens, numerous deficiencies exist that limit the effectiveness of biocontrol and biopreservation in food production. One major deficiency is that bacterial species such as *Pseudomonas fluorescens* have been found to cause a three log reduction of *Salmonella* in alfalfa sprouts; however, *Salmonella* was not completely eliminated from the alfalfa samples (Fett, 2006). This presents a serious deficiency in the case of food regulated under the Food and Drug Administration (FDA) as all *Salmonella spp.* are classified as adulterants under the Federal Food, Drug, and Cosmetic Act (FD&C); therefore, biocontrol cannot be utilized as a stand-alone procedure to protect foods from pathogens (FDA, 2012). As a result, biocontrol and biopreservation would need to be

incorporated into a hurdle technology approach to effectively mitigate pathogens. Hurdle technology is defined as the use of multiple control steps, "hurdles", to effectively eliminate pathogen contamination in a food manufacturing process (Singh & Shalini, 2016). In relation to traditional methods of pathogen control, Olanya et al. created a model that compares biocontrol and two types of chlorine treatments, a chlorine wash and chlorine dioxide, in a large scale and small scale tomato production setting. The results indicated that, in a large scale production setting, biocontrol is less expensive ($0.0058/kg) per kilogram of tomatoes produced when compared to chlorine dioxide treatments ($0.02/kg); however, the biocontrol treatment was more expensive than chlorine wash treatments ($0.00046/kg) (Olanya et al., 2016). Therefore, the economic cost of biocontrol must be weighed against that of traditional methods before biocontrol can be considered an effective replacement of traditional chemical treatments in the fruit and vegetable industry.

1.5 Understanding *Salmonella* Growth on Alfalfa (*Medicago sativa*) Sprouts

1.5.1 Current Knowledge of *Salmonella* Colonization and Growth on Alfalfa

When comparing various produce model systems to examine, alfalfa sprouts are ideal candidates mainly due to several factors: alfalfa sprouts have a rapid growth period (6-9 days), sprouts when compared to other produce such as tomatoes or lettuce have a smaller footprint in the laboratory, the amount of germinating sprouts can be changed easily to accommodate experiment demands, and sprouts do not require any special growth medium (Mohammad et al., 2020; NACMCF, 1999). In the literature, *Salmonella* has been found to colonize and grow on or in numerous parts of alfalfa sprouts. *Salmonella* mainly colonizes the surface of an infected seed or sprout with a higher cell density on the seed coat compared to other surfaces such as the root, cotyledon, and stem tissue (Cui et al., 2018). However, *Salmonella* has been found to internalize inside of sprout tissue through two different methods: chemotaxis into the stomata of the plant tissue when in the presence of light and colonization of lateral root cracks (Dong et al., 2003; Gandhi et al., 2001; Golberg et al., 2011).

Alfalfa is a leguminous plant that is known for forming close interactions with bacterial species in the rhizosphere i.e. the environment that is in close proximity to the plant's roots (Ramachandran et al., 2011; Wang et al., 2018). To promote the development of a bacterial community in the rhizosphere, plants produce and secrete compounds, exudates, such as carbohydrates, vitamins, and amino acids (Lukman et al., 2011). Lukman et al. performed gas chromatography-mass spectrometry on alfalfa seed exudate, and identified the following compounds: urea, glycerol, phosphate, homoserine, aspartate, fructose, glucose, galactonic acid, uric acid, and sucrose (Lukman et al., 2011). Based on this analysis, there is an abundant amount of carbon (glucose, glycerol, galactonic acid, fructose, and sucrose) and nitrogen (urea, uric acid, homoserine, and aspartate) sources that would support either the alfalfa's commensal microflora or *Salmonella*. According to Cui

et al., surface decontaminated alfalfa seeds inoculated with *Salmonella* at an initial inoculum of ~4 log CFU/g displayed rapid growth within 24 hours to 6.5 log CFU/g and the *Salmonella* cell density stabilized at 8 log CFU/g after 9 days of incubation (Cui et al., 2018). Therefore, *Salmonella* is highly capable of infecting alfalfa seeds and exponentially increasing in cell density when grown on a minimal-medium, water-agar, suggesting that the bacteria are being supported by exudates produced by the alfalfa.

Furthermore, a metagenomics study was conducted to determine the microfloral profile of store bought alfalfa sprouts. Jang et al. reported that the predominant bacteria associated with the sampled alfalfa sprouts were Enterobacteriaceae (33.6%), *Acinetobacter* (12.1%), *Janthinobacterium* (9.1%), and *Pseudomonas* (7.8%) (Jang et al., 2021). Enterobacteriaceae are a large family of Gram negative bacteria that includes non-pathogenic organisms, plant-associated organisms, coliform bacteria and human pathogens such as *Salmonella*, *Shigella* and *E. coli* (Jang et al., 2021; Metz et al., 2020; Walterson & Stavrinides, 2015). Although the high percentage of Enterobacteriaceae may have resulted from the manufacturing process of the store-bought alfalfa sprouts, the data would need to be stratified further to determine if organisms commonly associated with poor manufacturing hygiene conditions such as coliforms were the cause of this result. However, this result does indicate that members of Enterobacteriaceae such as *Salmonella* have been isolated from store-bought alfalfa sprouts and these organisms are able to outnumber bacterial species from other genera on the alfalfa.

1.5.2 Using "-Omics" Approaches to Study Genes Essential for *Salmonella* Growth on Alfalfa

To understand the close association that *Salmonella* has with alfalfa sprouts, a functional genomics, transcriptomics, or proteomics approach can be used to determine essential genes that are required for *Salmonella* to colonize/grow on alfalfa sprouts.

One methodology of functional genomics is the use of transposon insertion mutagenesis and subsequent sequencing to understand the impact of changes in a bacteria's genome on the fitness of that bacteria under experimental conditions (Lariviere & Batut, 2022). Transposons are highly regulated, short DNA segments that can excise themselves and translocate randomly within a genome (Lariviere & Batut, 2022). The random insertion of transposons would be used to generate mutants of *Salmonella* where the transposon has either inserted itself directly into a gene rendering it ineffective or the transposon has inserted itself into regulatory regions that will negatively impact a gene's expression (Davie et al., 2015; Lariviere & Batut, 2022). To do so, the transposon is inserted into *Salmonella* through the following methods: a plasmid encoding the transposon enters the host organism via electroporation or conjugation, or the transposon in association with a transposome enters the host organism via electroporation (Lee et al., 2013; Licandro-Seraut et al., 2012; Varushkina et al., 2021). Once a transposon mutant library is created of *Salmonella*, the mutant library can then be subjected to a particular growth condition such as being inoculated on alfalfa seeds (Lariviere & Batut, 2022). Since each individual

mutant will have at least one gene disrupted by a transposon, the diversity of the mutant library will change with respect to time i.e. less competitive mutants will be lower in abundance and more competitive mutants will have a higher abundance (Lariviere & Batut, 2022). To analyze the change in abundance of each mutant, polymerase chain reaction (PCR) and next-generation sequencing (NGS) would be used to amplify and subsequently sequence the transposon-genome junction (Lariviere & Batut, 2022). Based on the sequencing results, the relative abundance of each mutant can be cross-referenced to the initial abundance of mutants at the zero time point or the reference mutant library (Lariviere & Batut, 2022). Therefore, essential genes for growth in alfalfa can be correlated to mutants whose relative abundance decreased with respect to time because the loss of gene functionality conferred a negative fitness on that particular mutant.

One limitation of transposon insertion sequencing (TnSeq) is that only non-essential genes are studied simply because mutants with insertions in essential genes would not have survived the library preparation process (Cain et al., 2020). Additionally, transposon libraries must have an adequate amount of saturation i.e. the percentage of the genome that has a transposon insertion. DeJesus et al. notes that a saturation has to be high enough where each non-essential gene has at least several insertions; therefore, a saturation of 27 to 42% of a host's genome can be sufficient to perform TnSeq experiments (DeJesus et al., 2017). However, a high saturation of the host genome cannot guarantee that all non-essential genes will be completely knocked-out by transposon mutagenesis because *Salmonella* is known to possess genes that complement each other and the virulence plasmids of *Salmonella* have been found to have a heterogeneous copy number (Porwollik et al., 2014: Sánchez-Romero et al., 2020). Therefore, double or triple transposon mutants in the same or related genes would be required to effectively knock-out the function of a non-essential gene either on the chromosome or a virulence plasmid which would be statistically unlikely to occur during random transposon mutagenesis. As a result, TnSeq may accurately measure the effect of all non-essential genes in *Salmonella*.

In contrast, the use of transcriptomics does not determine the effects of gene knockouts on the overall fitness of an organism; rather, transcriptomics is used to provide a snapshot of the overall transcription of an organism's genes under experimental conditions (Lowe et al., 2017). Therefore, wild-type *Salmonella* would be inoculated on alfalfa seeds, multiple samples of the *Salmonella* cells would be collected over time, and the cells' RNA would be extracted (Lowe et al., 2017). Subsequently, the *Salmonella* RNA would be purified, and reverse transcriptase would be used to transcribe the single-stranded RNA into double-stranded cDNA (Lowe et al., 2017). The resulting cDNA could then be analyzed through two methodologies. One method would be the use of a DNA microarray that has short single-stranded DNA probes bound to a glass slide that correspond to various *Salmonella* genes, and when the probes bind the cDNA a fluorescent signal is produced (Lowe et al., 2017). Alternatively, RNA-seq could be performed in which NGS adapters would be attached to the 5' and 3' ends of the cDNA, and NGS would be performed to

map particular cDNA sequences to the *Salmonella* genome (Lowe et al., 2017). By using either method of cDNA analysis, the abundance of RNA transcripts can be determined at specific time points during the infection of *Salmonella* on alfalfa (Lowe et al., 2017). These results can then be compared to a reference transcriptome that is typically created from *Salmonella* grown in a liquid medium such as Luria-Bertani (LB) broth to determine the relative abundance of each transcript (Deng et al., 2012; Lowe et al., 2017). Therefore, RNA transcripts that have a higher relative abundance when compared to the reference transcriptome would identify potential genes that are required for the growth of *Salmonella* in alfalfa.

A limitation of RNA-seq can be attributed to the type of reference transcriptome that is used as a comparator for analysis. For example, Brankatschk et al. used the transcriptome of *Salmonella* that was grown in M9-minimal media as their reference transcriptome (Brankatshk et al., 2014). As a result, there may be subtle differences in the reference transcriptome compared to a reference transcriptome from the zero time point of an experiment that may provide valuable information. Additionally, RNA-seq experiments can be hindered due to technical difficulties such as: contamination of RNA-seq samples with RNA of off-target tissue i.e. plant tissue, the loss of RNA with each step in preparing RNA-seq samples that can lead to the loss of low abundance RNA, and changes in the transcriptome during the harvesting of bacterial cells (Heera et al., 2015; Marsh et al., 2017; Ozsolak & Milos, 2011).

Finally, proteomics can be utilized to understand how *Salmonella* is capable of growing in alfalfa. The proteome is a snapshot of the total intracellular and extracellular protein content of a cell (Aslam et al., 2017). Similar to a transcriptomics experiment, wild-type *Salmonella* would be inoculated on alfalfa seeds, multiple samples of the *Salmonella* cells would be collected over time, and the cells' protein would be extracted. Once a pure protein sample has been obtained, a wide variety of techniques can be performed to purify and analyze the extracted protein. One example of a high-throughput methodology is the use of liquid chromatography-tandem mass spectrometry (LC-MS/MS) where LC is used to fractionate the protein sample and MS/MS is used to determine the identity of each fractionated protein (Aslam et al., 2017). The abundance of each protein can be determined via spectral counts which represent the number of repeated peptide signals for each protein (Liu et al., 2015). Once the abundance of each protein is determined, the relative abundance of each protein can be determined by cross-referencing the sample against a reference proteome determined from *Salmonella* grown in a liquid culture (Liu et al., 2015). Therefore, proteins that are at a higher relative abundance compared to the reference proteome can be hypothesized to influence the ability of *Salmonella* to grow in alfalfa.

In the context of "-omics" approaches previously used to study the *Salmonella*-alfalfa relationship, RNA-seq and proteomic studies have been performed. Regarding transcriptomics, several studies have shown that *Salmonella* genes responsible for actions such as motility, biofilm formation, stress response, virulence, amino acid biosynthesis,

and antimicrobial resistance were elevated compared to the reference transcriptome (Brankatschk et al., 2014; Zheng et al., 2021). In terms of proteomic studies, Wang et al. has shown that proteins specifically responsible for amino acid biosynthesis were found in higher abundance compared to the reference proteome which further supports the same observation reported by Brankatschk et al.'s RNA-seq study (Brankatschk et al., 2014; Wang et al., 2015). Based on this result, the various "-omics' approaches can be utilized to corroborate and/or build on previous studies to complement each approach's flaws. Therefore, TnSeq, which has not been performed in alfalfa, could be used to further verify the aforementioned results and/or build upon our understanding of the ability of *Salmonella* to infect and colonize alfalfa sprouts.

1.5.3 Using Functional Genomics to Drive Biocontrol Agent Development

Ultimately, the goal of functional genomics, transcriptomics, and proteomics is to identify potential *Salmonella* genes that play a role in the infection of alfalfa; however, these broad-spectrum studies must be followed by classical genetics studies that target the identified genes to determine essentiality (Sawitzke et al., 2013). Therefore, a gene knockout must be performed in *Salmonella* where genetic engineering is utilized to cause an identified gene to lose all functionality, and a pure culture of the mutant is generated (Sawitzke et al., 2013). To test the hypothesis of a gene's essential function, the *Salmonella* mutant and wild-type *Salmonella* are inoculated into alfalfa, and the fitness of the mutant is compared to wild-type *Salmonella*. If the *Salmonella* mutant displays reduced fitness compared to the wild-type strain, then the gene that was knocked out can be considered essential for Salmonella to infect alfalfa. Furthermore, a rescue experiment can be performed where the essential gene located on a plasmid is restored in the mutant, and the rescued mutant and wild-type *Salmonella* are then inoculated in alfalfa (Barker et al., 2014). If the fitness of the rescued mutant is comparable to wild-type *Salmonella*, the gene is confirmed further to be essential towards the growth of *Salmonella* in alfalfa. Previous studies incorporating this classical genetics approach have identified *Salmonella* genes responsible for the following metabolic functions as essential to the infection of alfalfa: *de novo* amino acid biosynthesis, siderophore biosynthesis, attachment to plant cell walls, O-antigen biosynthesis, and the isomerization of fructose-6-phosphate to mannose-6-phosphate (Hao et al., 2012; Kwan et al., 2015; Kwan et al., 2018; Tan et al., 2016).

Using these results, the development of biocontrol agents can be further refined by screening biocontrol agents to target essential metabolites produced by either *Salmonella* or alfalfa that are critical for the infection of *Salmonella*. Kwan et al. have shown that the isomerization of fructose-6-phosphate to mannose-6-phosphate is critical for *Salmonella* to grow in alfalfa (Kwan et al., 2018). Therefore, a biocontrol agent can be screened for an enhanced metabolic consumption of fructose or mannose that would limit these compounds in alfalfa. Additionally, *de novo* amino acid biosynthesis was found to also play a significant role in the growth of *Salmonella* on alfalfa (Kwan et al., 2015). As a result, nitrogen sources such as urea, uric acid, homoserine, and aspartate that have been identified

in the exudate of alfalfa seeds would be potential metabolic targets for a biocontrol agent (Lukman et al., 2011). Therefore, the principle of this approach is to utilize data generated from broad-spectrum "-omics" studies to develop hypotheses of essential genes that can be tested using classical genetics, and the overall goal being the identification of exudates produced by alfalfa that can be targeted by biocontrol agents to limit the growth of *Salmonella*.

1.6 Conclusions

Salmonella contamination continues to be a salient public health issue because the incidence of *Salmonella* infections has not been reduced despite interventions from regulatory agencies and the food industry. Numerous thermal and non-thermal anti-microbial interventions have been developed to counter *Salmonella* contamination in sprouts; however, the application of these interventions have been hindered by factors such as the cost of the intervention; the efficacy of reducing the microbial load of treated products; and general consumer acceptability regarding non-natural processing methods. Bacterial biopreservation has been utilized by humans since the dawn of civilization. Furthermore, recent research has shown that certain biopreservation genera such as lactic acid bacteria provide positive health benefits when consumed, and plant-associated bacteria increase productivity of sprout growth. In comparison to chemical interventions, the production of bacterial biopreservation agents is more sustainable because waste by-products can be used as nutrient sources in their production. Criticism of bacterial biopreservation is centered around the cost of application compared to current treatment methods and the efficacy of reducing the pathogenic microbial load of a food sample. Although hundreds of bacterial biocontrol agents have been identified, the continued identification of potential biocontrol agents will further diversify the arsenal of biocontrol interventions to prevent further outbreaks. Furthermore, an "-omics" approach can be utilized to drive biocontrol agent development and target the metabolism of specific exudates that are critical towards the growth of foodborne pathogens in sprouts.

Based upon this literature review, two research goals were established. The first goal was focussed on isolating/characterizing plant-associated bacteria from alfalfa sprouts. Previous research has been performed extensively to understand the impact that a singular biocontrol agent has on controlling *Salmonella* cell density in sprouts; however, there has been a limited effort to understanding the effect that a community of biocontrol agents have upon *Salmonella* mitigation. To supplement the current gap in knowledge, a cocktail of plant-associated bacteria would be formulated to create a biocontrol cocktail, and the effectiveness of the cocktail would be assayed for the reduction of *Salmonella* contamination on alfalfa sprouts. Furthermore, the biocontrol cocktail would be assessed for the cocktail's impact on characteristics of the inoculated sprouts such as sprout biomass development and shelf-life following harvest.

The second goal was centered on utilizing a functional genomics approach through transposon insertion sequencing to determine *Salmonella* conditional gene essentiality to

the colonization and infection of alfalfa sprouts. Currently, a TnSeq study has yet to be performed using *Salmonella* on sprouts; therefore, this research goal would supplement our current understanding of *Salmonella* conditional gene essentiality that has previously been determined through studies utilizing transcriptomics, proteomics, and classical genetics. Furthermore, the use of TnSeq may allow for the identification of novel conditionally essential genes in the *Salmonella* genome that have yet to be elucidated. Following the identification of conditionally essential metabolic genes, metabolites produced by alfalfa or metabolites inherent to the sprout germination environment can be targeted for consumption by biocontrol agents. Therefore, the results of the transposon insertion sequencing study could then be used to guide the process of formulating a biocontrol cocktail specifically tailored towards the consumption of metabolite targets to ultimately decrease *Salmonella* cell density on alfalfa sprouts.

Chapter 2: Alfalfa (*Medicago sativa*) Sprout Infection Model System and Biocontrol Agent Development

2.1 Introduction

2.1.1 *Salmonella*: An Overview

Salmonella is a genus of Gram-negative, rod-shaped, and facultatively anaerobic bacteria belonging to the family Enterobacteriaceae (Eng et al., 2015). The World Health Organization has identified 2,579 serotypes of *Salmonella spp.* based on differences in the reactivity of *Salmonella* surface antigens such as the somatic (O) antigen and flagellar (H) antigen to anti-serums (Diep et al., 2019; Grimont & Weill, 2007). *S. enterica* subsp. *enterica* is the most prevalent subspecies of *Salmonella* comprising roughly 60 percent of the known serotypes of *Salmonella spp.* (Grimont & Weill, 2007). Additionally, *S. enterica* subsp. *enterica* (*Salmonella*) serotypes have been attributed to causing 99% of *Salmonella*-related infections in humans and warm-blooded animals (Jajere, 2019). However, a select number of serotypes of *Salmonella* are responsible for the majority of infections. According to Jackson et al., a Center for Disease Control and Prevention (CDC) study in 2009 discovered that only 20 serotypes of *Salmonella* were responsible for over 82 percent of the approximately 36,000 *Salmonella* isolates collected from reported human infections in that year (Jackson et al., 2013).

2.1.2 Impact of *Salmonella* on the General Population and the Food System in the United States

Salmonellosis is a gastrointestinal infection with key symptoms of diarrhea (with or without blood), fever, and stomach cramps; however, other symptoms such as nausea, vomiting, and/or stomach cramps may be observed (CDC: Symptoms, 2019). Cases of salmonellosis present within 8 to 72 hours post-infection, and the infection persists for several days for the average person (Mayo Clinic: Salmonella Infection, 2019). In the United States (US), *Salmonella* is responsible for an estimated 1.2 million illnesses each year (Jackson et al., 2013). Although cases of salmonellosis vary in severity depending on the specific serotype of *Salmonella* and the health of the infected person; children under the age of 5, the elderly, and immunocompromised people are highly susceptible to *Salmonella* infections compared to the average healthy person (Eng et al., 2015). Therefore, the aforementioned populations would be more susceptible to developing complications such as severe dehydration, bacteremia (presence of bacteria in the bloodstream), and reactive arthritis (Mayo Clinic, 2019). Salmonellosis normally passes without the need for additional treatments except for the intake of fluids and electrolytes (CDC: Diagnosis & Treatment, 2019). However, in severely complicated cases, antibiotics are commonly administered to clear the bacteria (Mayo Clinic, 2019). *Salmonella* is ubiquitous in the environment and natural reservoirs such as poultry, cattle, swine, and wild birds have been documented (Jarere, 2019).

In the US, greater than 75% of *Salmonella* illnesses were attributed to the following seven product categories: chicken, fruits, pork, seeded vegetables, other produce such as nuts, turkey, and eggs (Interagency Food Safety Analytics Collaboration, 2021). Furthermore, products such as beef, vegetable row crops, sprouts, dairy, and fish contributed to the remaining 25% of attributed *Salmonella* illnesses (Interagency Food Safety Analytics Collaboration, 2021). Of the 1,531 serotypes of *Salmonella*, four particular serotypes have been identified as key epidemiologically important serotypes in foodborne illness: *Salmonella* Typhimurium, Enteritidis, Heidelberg, and Newport (Jarere, 2019). These four serotypes were responsible for ~350 outbreaks between 2007 and 2011 in the US; however, these serotypes remain largely restricted to certain food categories (Andino & Hanning, 2015). *Salmonella* Enteritidis is commonly associated with animal products such as eggs, chicken, pork, and beef. *Salmonella* Typhimurium is associated with chicken, leafy greens, and peanut butter. *Salmonella* Heidelberg is associated with chicken, turkey, and dairy products. *Salmonella* Newport is associated with sprouts, vegetables, tomatoes, pork, and poultry (Andino & Hanning, 2015). Despite enacted regulations in the US, the relative rate of annual *Salmonella* infections remained the same from 2008 to 2014 (Crim et al., 2015). Thus, the US continues to be afflicted with *Salmonella* leading to an annual economic burden estimated at $4.14 billion as of 2020 (USDA & ERS, 2020).

2.1.3 Sprouts and *Salmonella*

Within the produce category, sprouts were attributed with causing 4.2 percent of *Salmonella*-related foodborne illnesses (Interagency Food Safety Analytics Collaboration, 2021). Sprouts have progressively gained a greater consumer interest in Western cultures since the 1980's due to a higher demand for less-processed produce with fewer additives that can be used as garnishes on salads, sandwiches, or other high-quality foods (Benincasa et al., 2019). Furthermore, sprouting is an inexpensive process that occupies a minimal amount of greenhouse space, has a short growth cycle to reach harvest maturity, requires minimal processing equipment, and results in relatively high yields of sprouts (Kyriacou et al., 2016; Lorenz & D'Appolonia, 2009). According to the National Advisory Committee on Microbiological Criteria for Foods (NACMCF), sprouts are grown in a dark, humid environment for an average of 3 to 7 days at 21 to 26°C, and the sprouting environment varies from plastic 5-gallon pails, 32-gallon barrels, sprouting beds, or growth chambers optimized for sprout production (NACMCF, 1999). However, sprouting conditions such as time, temperature, water activity, pH, and available nutrients have been found to promote the growth of foodborne pathogens such as *Salmonella* (FDA, 2017). As a result, there have been twenty-two *Salmonella*-related outbreaks between 1999 and 2020 recorded in the United States that have resulted in 1003 reported illnesses (Miyahira & Antunes, 2021). Furthermore, the contamination of sprouts resulted in a high-profile case where the Food and Drug Administration (FDA) filed a warning letter to the US-based sandwich chain Jimmy John's due to five independent outbreaks of *Salmonella* and *Escherichia coli* that were associated with sprouts served at several Jimmy John's locations

(FDA 2020). The warning letter stated that Jimmy John's had not taken significant corrective measures to prevent the sale of adulterated sprouts in their stores despite repeated interventions over an eight year period of time (FDA, 2020). Therefore, sprouts present a rapidly expanding market with increased consumer interest that is highly susceptible to contamination by foodborne pathogens such as *Salmonella* that has led to numerous multistate outbreaks of Salmonellosis.

2.1.4 Current Microbial Interventions in Sprouts and Criticisms

According to the NACMCF, sources of contamination of sprouts can be traced to improperly treated water, animal waste, poor sanitation of equipment, and/or poor personal hygiene of workers; however, sprouting seeds are likely to be the main source of contamination (NACMCF, 1999). Chemical treatments using compounds such as chlorine, acetic acid, or calcium hypochlorite are typically used to decontaminate the surface of seeds resulting in an average reduction of 2 logs (Mir et al., 2021). However, research has shown that methods such as acidic electrolyzed water, cold plasma, high pressure processing, hot water, ultrasound, pulsed light, ultraviolet irradiation, and gamma irradiation have also been used to decontaminate the seed surface (Mir et al., 2021; Puligundla et al., 2017; Wuytack et al., 2003). The efficacy of these various treatments varies quite dramatically with hot water and gamma irradiation achieving 6 to 7 log reductions, respectively, whereas ultraviolet irradiation was found to only achieve a ~1 log reduction (Mir et al., 2021).

Another intervention occurs after the sprouts have been harvested. Chemical treatments, acidic electrolyzed water, cold plasma, gamma irradiation, and ultraviolet irradiation have been studied as potential post-harvest intervention methods (Mir et al., 2021). Similar to seed decontamination, chemical treatments resulted in a 2 log reduction of the background microflora on the treated sprouts (Mir et al., 2021). Oh et al. reported that the use of Nitrogen cold plasma resulted in a 2.6 log reduction of *Salmonella* Typhimurium on the surface of radish sprouts (Oh et al., 2017). Finally, 2 kGray of gamma irradiation was reported to cause a 5 log reduction of *Salmonella* in alfalfa, mung bean, and radish sprouts (Rajkowski & Thayer, 2000).

Although there are numerous methods that have been developed to control *Salmonella* in sprouts, each method may not be effective or practical due to each technology's own limitations. In terms of chemical rinses and ultraviolet irradiation being used to decontaminate the surface of seeds, these interventions lead to a 1 to 2 log reduction in *Salmonella*; however, the NACMCF recommends a 5 log reduction to effectively eliminate *Salmonella* from the seed surface (Mir et al., 2021; NACMCF, 1999). Additionally, methods such as electrolyzed oxidized water, ultraviolet light, cold plasma, and chemical washes are limited to the surface of a treated product (Mukhopadhyay & Ramaswamy, 2012). These surface level treatments present a severe limitation because *Salmonella* is known to internalize inside of the sprout tissue (Dong et al., 2003; Gandhi et al., 2001; Golberg et al., 2011). As a result, internalized *Salmonella* would be impervious

to surface-level treatments. Although gamma irradiation showed promising results in reducing *Salmonella* by 5 to 6 logs in finished sprouts and seeds, respectively, irradiation of foods continues to have an unfavorable public perception (Mir et al., 2021; Shen et al., 2017). Based upon these criticisms, there is a high demand for continued innovation of current control methods and the discovery of novel control methods of *Salmonella* in sprouts is imperative.

2.1.5 Biocontrol as a Potential Alternative

One potential solution to mitigating *Salmonella* contamination in sprouts can be done through biocontrol i.e. the introduction of a natural or genetically modified organism to a system to manage the population of undesirable and/or harmful pests (Thakur & Singh, 2018). In the context of biocontrol in sprouts, the application of plant-associated bacterial species has experienced substantial attention because these organisms often have a pre-existing commensal relationship with the plant and these bacterial species can reach high cell densities upwards of 10^9 CFU/g of plant tissue (Gnanamanickam, 2006). Furthermore, plant-associated bacterial species such as *Pseudomonas* and *Bacillus* are well-known to produce a myriad of antibiotics that can have a detrimental effect on other microorganisms such as *Salmonella* (Beneduzi et al., 2012; Raaijamakers et al., 2002).

In alfalfa sprouts, *Pseudomonas fluorescens* strain 2-79 was found to cause a 5.5 log reduction of *Salmonella* within a 24 hour time span of seed germination (Fett, 2005). Although this result is quite promising, alfalfa sprouts are incubated for 5-7 days prior to harvesting and the final *Salmonella* cell density was only reduced to ~4.5 log CFU/g (Fett, 2005; NACMCF 1999). *Erwinia persicina* strain EUS78 when applied to alfalfa sprouting seeds at an initial cell density of 5 log CFU/g led to only a 2.5 log reduction of *Salmonella* after 6 days of incubation (Kim et al., 2020). However, when the inoculum of *E. persicina* EUS78 was increased to 8 log CFU/g, *Salmonella* was completely eliminated from the alfalfa sprouts after 6 days of incubation (Kim et al., 2020). Therefore, a plant-associated bacterial biocontrol agent may not be effective in completely eliminating *Salmonella* when applied at a relatively low cell density; however, increasing the cell density of the biocontrol agent may increase the biocontrol agent's effectiveness against *Salmonella*. In terms of addressing internalized *Salmonella*, *Bacillus subtilis* was found to have a significant effect on *Salmonella* internalization resulting in a 1 to 1.5 log reduction of internalized *Salmonella* after 5 days of incubation (Shen et al., 2017). In contrast to experiments using a biocontrol agent monoculture, Matos & Garland isolated a community of plant-associated bacteria from store-bought alfalfa sprouts composed of *Pseudomonas spp., Pantoea spp., Erwinia rhapontici, Escherichia hermannii, Stenotrophomonas maltophila, Acinetobacter spp.,* and *Enterobacter pyrinus* (Matos & Garland, 2005). Using this community of microorganisms, a 6 log reduction of *Salmonella* was observed and *Salmonella* was nearly eliminated from the inoculated alfalfa sprouts after 7 days of incubation (Matos & Garland, 2005).

In summation, biocontrol can be an effective control strategy of *Salmonella* in alfalfa sprouts. However, biocontrol has also been found to not completely eliminate *Salmonella* from alfalfa sprouts, thus sprouts with a reduced amount of *Salmonella* would still be in violation of the Produce Rule (Produce Rule, 2015). As a result, biocontrol should not be used as the only control measure to limit *Salmonella* contamination; rather, biocontrol should be implemented to work in synchrony with seed decontamination and post-harvest mitigation strategies in a hurdle approach to sprout safety.

2.1.6 Selection of Alfalfa as a Model System

When comparing various produce model systems to examine, alfalfa sprouts are ideal candidates mainly due to several factors: alfalfa sprouts have a rapid growth period (6-9 days), sprouts when compared to other produce such as tomatoes or lettuce have a smaller footprint in the laboratory, the amount of germinating sprouts can be changed easily to accommodate experiment demands, and sprouts do not require any special growth medium (Mohammad et al., 2020; NACMCF, 1999). In the literature, *Salmonella* has been found to colonize and grow on or in numerous parts of alfalfa sprouts. *Salmonella* mainly colonizes the surface of an infected seed or sprout with a higher cell density on the seed coat compared to other surfaces such as the root, cotyledon, and stem tissue (Cui et al., 2018). However, *Salmonella* has also been found to internalize inside of sprout tissue (Dong et al., 2003; Gandhi et al., 2001; Golberg et al., 2011). According to Cui et al., surface decontaminated alfalfa seeds inoculated with *Salmonella* at an initial inoculum of ~4 log CFU/g displayed rapid growth within 24 hours to 6.5 log CFU/g and the *Salmonella* cell density stabilized at 8 log CFU/g after 9 days of incubation (Cui et al., 2018). Therefore, *Salmonella* is highly capable of infecting alfalfa seeds and exponentially increasing in cell density when grown on a minimal-media, water-agar, suggesting that the bacteria are being supported by exudates produced by the alfalfa.

Furthermore, a metagenomics study was conducted to determine the microfloral profile of store bought alfalfa sprouts. Jang et al. reported that the predominant bacteria associated with the sampled alfalfa sprouts were Enterobacteriaceae (33.6%), *Acinetobacter* (12.1%), *Janthinobacterium* (9.1%), and *Pseudomonas* (7.8%) (Jang et al., 2021). Enterobacteriaceae are a large family of Gram negative bacteria that includes non-pathogenic organisms, plant-associated organisms, coliform bacteria and human pathogens such as *Salmonella*, *Shigella* and *E. coli* (Jang et al., 2021; Metz et al., 2020; Walterson & Stavrinides, 2015). Although the high percentage of Enterobacteriaceae may have resulted from the manufacturing process of the store-bought alfalfa sprouts, the data would need to be stratified further to determine if organisms commonly associated with poor manufacturing hygiene conditions such as coliforms were the cause of this result. However, this result does indicate that members of Enterobacteriaceae such as *Salmonella* have been isolated from store-bought alfalfa sprouts and these organisms are able to outnumber bacterial species from other genera on alfalfa.

2.1.7 Objectives

The overall goal of this research was focussed upon isolating either a single plant-associated bacterial species or a community of plant-associated bacterial species that would be an effective biocontrol measure against *Salmonella* in alfalfa sprouts. To achieve this goal, the following seven objectives were established:

1. Develop a seed decontamination protocol that effectively eliminates the background microflora from purchased alfalfa seeds to mitigate the impact of this microflora on biocontrol agent inoculation, *Salmonella* inoculation, or biocontrol agent-*Salmonella* co-inoculation experiments.
2. Isolate plant-associated bacteria from alfalfa seeds/sprouts.
3. Characterize these plant-associated bacteria based on attributes such as colony morphology, Gram stain, cell morphology, and ability to grow on a selective-medium specific to *Salmonella*. Identify isolates based upon 16S rRNA gene sequencing.
4. Assay prospective plant-associated bacterial isolates' ability to positively influence alfalfa sprout biomass production and attain a high cell density *in planta*.
5. Develop a cocktail of plant-associated bacteria, and assay the cocktail's effect on alfalfa sprout biomass development and shelf-life of alfalfa sprouts under refrigerated conditions.
6. Utilize whole genome sequencing to identify each constituent of the plant-associated bacterial cocktail and assay each constituent's genome for genes associated with plant pathogenicity.
7. Determine the efficacy of the plant-associated bacterial cocktail in mitigating *Salmonella* contamination in alfalfa sprouts.

2.2 Materials & Methods

2.2.1 Seed Surface Decontamination and Growth Conditions for Alfalfa Sprouts

Two brands of alfalfa seeds were used to evaluate a seed surface decontamination protocol: International Specialty Supply (ISS) and Food to Live (FTL). The ISS seeds were purchased directly from the seed distribution company. The FTL seeds were purchased on Amazon.com via the FTL company page.

2.5 grams of alfalfa seeds (ISS or FTL) were added to a sterile 250 mL flask. To remove loosely bound particles on the seed surface, 250 mL of distilled water (dH_2O) with approximately 0.5 mL of Tween-20 was added to the flask, and the flask was shaken at 200 RPM for 5 minutes to distribute the alfalfa seeds in the rinse mixture. Tween-20 is a surfactant that was used to remove loosely bound particles or biofilms from the seed surface that would prevent the 95% ethanol and 20% bleach used in the decontamination process to effectively eliminate the background microflora present on the seed surface. The dH_2O-Tween-20 solution was then decanted into a waste bucket, and 250 mL of 95% ethanol was added to the flask and returned to the shaker for another 5 minutes. The 95%

ethanol was then decanted into a waste bucket, and a 10-20% solution of commercial bleach (4.5% sodium hypochlorite, 4.275% available chlorine) was added to the flask. The flask was then shaken for 20 minutes, and the bleach solution was decanted into a waste bucket. To remove any excess chlorine from the seed surface, the decontaminated alfalfa seeds were rinsed six times with 400 mL of sterile dH_2O. To measure the percent germination and percent contamination of the decontaminated alfalfa seeds, ~100-200 seeds were aseptically transferred to Reasoner's 2A agar (R2A) plates (Lee et al., 2016). The R2A plates were then incubated at 25°C for 48 hours. After incubation, the number of alfalfa seeds that displayed signs of germination and contamination were counted. The resulting number of germinated seeds and contaminated seeds was then divided by the total number of seeds on the R2A plate to yield a percent germination and percent contamination.

To allow the germination and growth of alfalfa sprouts, surface decontaminated seeds were incubated in the dark at 25°C. An incubation environment for sprouting was achieved either on a large-scale or small-scale basis. At a large-scale, 10 to 15 grams of surface decontaminated seeds were added to a sterile Pyrex® dish containing approximately 400 mL of 1.0% water-agar, and a sealed environment was created using a Sun bag (Millipore-Sigma, Burlington, MA) with a 0.02 μm filter to allow for gas-exchange (Figure 1A) (Cui et al., 2018). For small-scale experiments, 2.5 to 5 grams of surface decontaminated seeds were added to sterile Pyrex® petri dishes containing approximately 40 mL of 1.0% water-agar (Cui et al., 2018). Additionally, a secondary sterile Pyrex® petri dish was added to the system and filled with sterile dH_2O to humidify the sprouting environment. To create a sealed environment, the petri dishes were incubated in large glass dishes (Figure 1B).

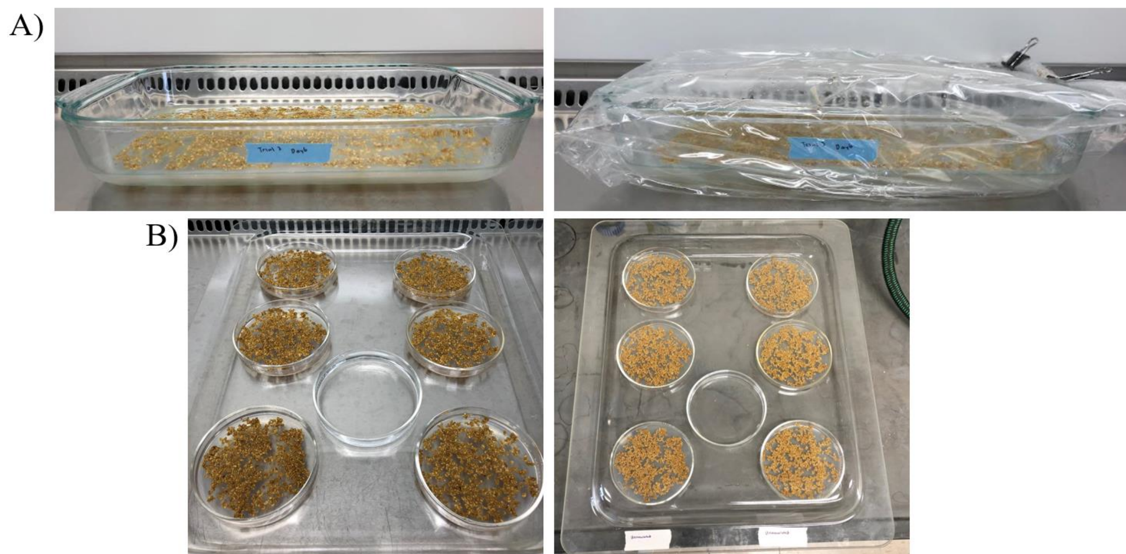

Figure 1A/B: Photographs of the large-scale (A) and small-scale (B) environments used to achieve sprouting conditions. Large Pyrex® dishes or Pyrex® petri dishes were filled with 1.0% water-agar to provide a source of hydration for the sprouts.

2.2.2 Isolation of Plant-Associated Bacteria

To isolate plant-associated bacteria (PAB) from the surface of alfalfa plant tissue, four 10 gram aliquots of surface decontaminated or untreated seeds were grown under large-scale growth conditions for six days. At the 0, 1, 3, and 6 day time points, the alfalfa seeds and/or sprouts were harvested, transferred into sterile flasks, and plant-associated bacterial cells were extracted from the plant surface. To isolate the bacterial cells, approximately 25 to 400 mL of PHG solution (0.1% Bacto-Gelatin and 0.1 M $(NH_4)_2HPO_4$) was added to the flask to completely immerse the plant tissue, then 100 5 mm glass beads were added to the flask to homogenize the plant tissue (Kingsley & Bohlool, 1981). The flasks were then shaken at 200 RPM for 30 minutes. With volumes in excess of 50 mL of PHG solution, the resulting PAB extract was transferred to a sterile 400 mL Bio-Bottle (ThermoFisher, Waltham, MA) and centrifuged at 5,000 RPM for 15 minutes. The supernatant was discarded into biological waste; 25 mL of PBS (137 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, and 2 mM KH_2PO_4) was added to the Bio-Bottle and vortexed; and the concentrated bacterial sample was transferred to a sterile 50 mL Falcon tube. For volumes less than or equal to 50 mL of PHG solution, the resulting PAB extract was transferred directly to a sterile 50 mL Falcon tube. From the PAB extract, a serial dilution was performed to yield a 10^{-1}, 10^{-3}, 10^{-5}, and 10^{-7} dilution. R2A and Lysogeny Broth (LB) agar plates were spread-plated using the prepared dilutions, and the plates were incubated at 25°C for 48 hours and at 37°C for 24 hours, respectively.

After incubation, the colony forming units (CFU) were counted on the LB and R2A plates, the CFU/mL of the PAB extract was determined, and the CFU/g of the PAB from the isolated alfalfa was determined using the measured volume of PAB extract and mass of alfalfa harvested.

PAB isolates were identified on the R2A and LB plates, and a sterile inoculating loop was used to streak each PAB isolate on LB for single colonies. The streaked LB plates were incubated at 30°C for 24 hours. The process was then repeated to obtain pure PAB isolates. To prepare the PAB isolates for extended storage, liquid cultures of the isolates were prepared by inoculating a single PAB isolate colony into 5 mL of liquid LB and incubating at 30°C with shaking for 24 hours. After incubation, the liquid cultures were transferred to 15 mL Falcon tubes and centrifuged at 5000 RPM. The liquid was decanted into biological waste, the cell pellets were resuspended in 1 mL of LB containing 25% glycerol v/v, and the PAB isolates were stored at -80°C until further analysis.

2.2.3 Characterization and Identification of Plant-Associated Bacteria

To further characterize the PAB isolates, each isolate was recovered from -80°C storage by streaking the isolate on an LB agar plate, and incubated at 30°C for 24 hours. Once single colonies were observed to be growing on the surface of the LB plate, the colony morphological characteristics were recorded. Then a single colony was aseptically transferred to a glass microscope slide, heat fixed on the slide surface, and a Gram stain was performed. The bacteria were then visualized under a light microscope at 1000X

magnification using oil immersion. Once the bacteria were visualized, characteristics such as the Gram staining characteristic, cell shape, and relative cell size were recorded. To screen for potential *Salmonella* isolates, each PAB isolate was streaked on Xylose-Lysine-Tergitol 4 (XLT4) agar plates and incubated at 37°C for 24 hours. After incubation, colonies that displayed growth on XLT4 and had black centers were recorded as presumptive *Salmonella* isolates.

To identify the PAB isolates, a colony polymerase chain reaction (PCR) approach was taken to amplify the 16S ribosomal rRNA gene sequence. Rather than perform an extraction of the genomic DNA of each PAB isolate, a single colony of each isolate was aseptically transferred to 50 µL of nuclease-free water and the sample was vortexed to resuspend the bacteria. Using the primers outlined in Table 1, PCR reactions were formulated according to Table 2 and PCR was performed following the thermal cycler conditions outlined in Table 3.

Table 1: Primers used to amplify the 16s rRNA gene sequence in the PAB isolates.

Primer Name	Primer Sequence	Reference
V1F	5'-AGAGTTTGATCCTGGCTCAG-3'	Ritschard et al., 2018
V8R	5'-GACGGGCGGTGWGTRC-3'	Ritschard et al., 2018

Table 2 and Table 3: PCR reaction components (Table 2) and the PCR Reaction conditions used to amplify the 16S rRNA gene sequence in the PAB isolates (Table 3).

Reaction Preparation		PCR Conditions		
Reagent	Volume (µL)	Temperature	Number of PCR Cycles	Time
PAB Isolate Mix	1.0	95°C	1	1 min
V1F (10 µM)	1.0	95°C		15 sec
V8R (10 µM)	1.0	50°C	30	30 sec
Taq 2x Master Mix	25	72°C		30 sec
Nuclease-Free H_2O	22	72°C	1	5 min
Total	50	4°C	1	Hold

Following PCR, each reaction was cleaned-up using the Zymo Clean and Concentrator-5 kit (Zymo Research, Irvine, CA), and the concentration of double-stranded (ds) DNA was determined using a Qubit fluorometer (ThermoFisher, Waltham, MA). The concentration of the dsDNA was then normalized to 2 ng/µL (30 ng dsDNA per 15 µL nuclease-free water), 25 pmol (2.5 µL) of the 10 µM V1F primer was added to the dsDNA, and the samples were sent to Genewiz (Azenta Life Sciences, Plainfield, NJ) for Sanger sequencing to be performed. Once Sanger sequencing was complete, the sequencing results were visualized via a chromatogram and a DNA sequence was determined from the basecalls using the ApE software package (Davis & Jorgensen, 2022). Then the Basic Local Alignment Search Tool (BLAST) provided through the National Center for Biotechnology Information (NCBI) was used to search the center's database for homologous sequences to those determined from the Sanger sequencing. Based on the nucleotide BLAST results, PAB isolates were identified either at the genus or the species level.

2.2.4 Plant-Associated Bacteria Inoculation

Overnight cultures of each PAB isolate were prepared by inoculating 5 mL of LB broth and incubating at 30°C with shaking. Four 2.5 gram aliquots of surface decontaminated seeds were inoculated to an initial inoculum of 4 log CFU/g PAB isolate. A negative control composed of four 2.5 gram aliquots of surface decontaminated seeds that were not inoculated with PAB were also prepared. This negative control was repeated 5 times. The inoculated and uninoculated seeds were then grown under small-scale growth conditions for six days. At the 0, 1, 3, and 6 day time points, the uninoculated seeds and/or sprouts were transferred to a weight boat, their biomass was recorded, and the plant tissue was then discarded. At the same time points, the PAB inoculated alfalfa seeds and/or sprouts were harvested, transferred into sterile flasks, and the PAB cells were extracted from the plant surface. To isolate the bacterial cells, approximately 5 to 50 mL of PHG solution was added to the flask to completely immerse the plant tissue, and approximately 50 5 mm glass beads were added to each flask (Kingsley & Bohlool, 1981). Then, the flasks were shaken at 200 RPM for 30 minutes. The resulting PAB extract was transferred directly to a sterile 50 mL Falcon tube. A serial dilution was performed to yield a 10^{-1}, 10^{-3}, 10^{-5}, and 10^{-6} dilution. LB agar plates were spread-plated using the prepared dilutions, and the plates were incubated at 30°C for 24 hours.

After incubation, the CFUs were counted on the LB plates, the CFU/mL of the PAB extract was determined, and the CFU/g of PAB from the isolated alfalfa was determined using the measured volume of extract and mass of alfalfa harvested.

2.2.5 Biocontrol Agent Cocktail: Biomass Development and Shelf-life Study

Overnight cultures of each PAB cocktail constituent were prepared by inoculating 5 mL of LB broth and incubated at 30°C with shaking (Table 4).

Table 4: Constituents of the plant-associated bacterial biocontrol cocktail and source of isolate.

Isolate	Source
Pantoea agglomerans (JV6)	FTL Alfalfa Seeds/Sprouts
Priestia megaterium #3 (JV24)	ISS Alfalfa Seeds/Sprouts
Pseudomonas sp. #1 (RG1)	Store-Bought Alfalfa Sprouts
Pantoea sp. #3 (RG18)	Store-Bought Alfalfa Sprouts
Pseudomonas putida KT-2440	Dr. Michael Sadowsky

Four 5 gram aliquots of surface decontaminated seeds were inoculated to an initial inoculum of 7 log CFU/g of the PAB cocktail. This was then repeated for a total of two replicates. A negative control composed of four 5 gram aliquots of surface decontaminated seeds that were not inoculated with PAB were also prepared. This negative control was repeated 3 times. The inoculated and uninoculated seeds were then grown under small-scale growth conditions for six days. At the 0, 1, 3, and 6 day time points, the uninoculated and inoculated seeds and/or sprouts were transferred to a weight boat, their biomass was recorded, and the plant tissue was then discarded. At the same time points, one replicate of the PAB inoculated alfalfa seeds and/or sprouts were harvested, transferred into sterile flasks, and the PAB cells were extracted from the plant surface. To isolate the bacterial cells, approximately 5 to 50 mL of PHG solution was added to the flask to completely immerse the plant tissue, and approximately 50 5 mm glass beads were added to each flask (Kingsley & Bohlool, 1981). Then, the flasks were shaken at 200 RPM for 30 minutes. The resulting PAB extract was transferred directly to a sterile 50 mL Falcon tube. A serial dilution was performed to yield a 10^{-1}, 10^{-3}, 10^{-5}, and 10^{-6} dilution. LB agar plates were spread-plated using the prepared dilutions, and the plates were incubated at 30°C for 24 hours.

After incubation, the CFUs were counted on the LB plates, the CFU/mL of the PAB extract was determined, and the CFU/g of PAB from the isolated alfalfa was determined using the measured volume of extract and mass of alfalfa harvested.

A shelf-life study was performed using one day 6 PAB inoculated alfalfa sample and three day 6 uninoculated alfalfa samples. After the biomass was determined, the alfalfa sprouts were transferred to sterile 100mm x 25mm petri dishes. Qualitative characteristics

of the sprouts such as color, rigidity, presence of exudate, size, and general signs of spoilage were recorded. The sprouts were then stored at 4°C for a period of two weeks, and the aforementioned characteristics were recorded every three days.

2.2.6 Biocontrol Agent Cocktail: Whole Genome Sequencing

To identify the specific genus, species, and strain of each constituent of the PAB cocktail, whole genome sequencing was utilized. Overnight cultures of each PAB cocktail constituent were prepared by inoculating 5 mL of LB broth and incubated at 30°C. The genomic DNA (gDNA) of each PAB cocktail constituent was then extracted using the NucleoSpin Tissue kit (TakaraBio, Kusatsu, Shiga, Japan) following the manufacturer's instructions. After extraction, the concentration of dsDNA was determined using a Qubit fluorometer (ThermoFisher, Waltham, MA). 30 µL (> 10 ng/µL) of each PAB cocktail constituent's gDNA was then shipped to the Microbial Genome Sequencing Center (MiGS, Pittsburgh, PA) for whole genome sequencing (WGS) using the 400 Mbp sequencing package offered through MiGS. Once WGS was completed, the output files were downloaded and uploaded to the Bacterial and Viral Bioinformatics Resource Center (BV-BRC, Chicago, IL). The raw sequencing reads were then processed using the Comprehensive Genome Analysis service offered through BV-BRC to assemble the raw sequencing reads into a draft genome, assay the quality of the draft genome, and annotate the draft genome with known and hypothetical genes (BV-BRC, https://www.bv-brc.org/). Following the Comprehensive Genome Analysis, the genomes were viewed in BV-BRC using the genome Browser/Circular Viewer feature and each genome was queried for genes associated with plant pathogenicity using the Proteins feature of the BV-BRC website (BV-BRC, https://www.bv-brc.org/). To identify each PAB cocktail constituent to the species level, the assembled PAB genomes were queried against reference, representative, and public genomes of *Pantoea spp.*, *Priestia spp.*, or *Pseudomonas spp.* available on BVBRC using the Phylogenetic Tree Building service (BV-BRC, https://www.bv-brc.org/).

2.2.7 *Salmonella* Typhimurium 4/74 Inoculation

An overnight culture of *Salmonella* Typhimurium 4/74 was prepared by inoculating 5 mL of LB broth and incubated at 37°C with shaking. Four 2.5 gram aliquots of surface decontaminated or untreated seeds were inoculated in triplicate to an initial inoculum of 4 log CFU/g *Salmonella* Typhimurium 4/74. The inoculated seeds were then grown under small-scale growth conditions for six days. At the 0, 1, 3, and 6 day time points, the alfalfa seeds and/or sprouts were harvested, transferred into sterile flasks, and the *Salmonella* Typhimurium 4/74 cells were extracted from the plant surface. To isolate the bacterial cells, approximately 5 to 50 mL of PHG solution was added to the flask to completely immerse the plant tissue, and approximately 50 5 mm glass beads were added to each flask (Kingsley & Bohlool, 1981). Then, the flasks were shaken at 200 RPM for 30 minutes. The resulting *Salmonella* Typhimurium 4/74 extract was transferred directly to a sterile 50 mL Falcon tube. A serial dilution was performed to yield a 10^{-1}, 10^{-3}, 10^{-5}, and 10^{-6} dilution. XLT4

agar plates were spread-plated using the prepared dilutions, and the plates were incubated at 37°C for 24 hours.

After incubation, the CFUs were counted on the XLT4 plates, the CFU/mL of the *Salmonella* Typhimurium 4/74 extract was determined, and the CFU/g of *Salmonella* Typhimurium 4/74 from the isolated alfalfa was determined using the measured volume of extract and mass of alfalfa harvested.

2.2.8 *Salmonella*-Biocontrol Agent Coinfection

Overnight cultures of *Salmonella* Typhimurium 4/74 and each PAB cocktail constituent were prepared by inoculating 5 mL of LB broth and incubated at 37°C and 30°C with shaking for *Salmonella* Typhimurium 4/74 and the PAB, respectively (Table 4). Four 5 gram aliquots of surface decontaminated seeds were inoculated in triplicate to an initial inoculum of 2 log CFU/g *Salmonella* Typhimurium 4/74 and 7 log CFU/g of the PAB cocktail. This process was performed in triplicate. The inoculated seeds were then grown under small-scale growth conditions for six days. At the 0, 1, 3, and 6 day time points, the alfalfa seeds and/or sprouts were harvested, transferred into sterile flasks, and the *Salmonella* Typhimurium 4/74 cells were extracted from the plant surface. To isolate the bacterial cells, approximately 5 to 50 mL of PHG solution was added to the flask to completely immerse the plant tissue, and approximately 50 5 mm glass beads were added to each flask (Kingsley & Bohlool, 1981). Then, the flasks were shaken at 200 RPM for 30 minutes. The resulting *Salmonella* Typhimurium 4/74 extract was transferred directly to a sterile 50 mL Falcon tube. A serial dilution was performed to yield a 10^{-1}, 10^{-3}, 10^{-5}, and 10^{-6} dilution. XLT4 agar plates were spread-plated using the prepared dilutions, and the plates were incubated at 37°C for 24 hours.

After incubation, the CFUs were counted on the XLT4 plates, the CFU/mL of the *Salmonella* Typhimurium 4/74 extract was determined, and the CFU/g of *Salmonella* Typhimurium 4/74 from the isolated alfalfa was determined using the measured volume of extract and mass of alfalfa harvested.

2.3 Results & Discussion

2.3.1 Seed Surface Decontamination

As stated in the sprouts provision of the Produce Rule, measures must be taken to ensure that sprouting seeds are decontaminated prior to germination to ensure pathogen reduction (Produce Rule, 2015). Additionally, seed surface decontamination is necessary to eliminate the presence of naturally occurring commensal bacterial species inhabiting the seed surface (López et al., 2018; Jang et al., 2021). Therefore, the development of an effective seed decontamination procedure was required to ensure that downstream experiments such as biocontrol agent inoculations, *Salmonella* inoculations, or biocontrol agent-*Salmonella* co-inoculations of alfalfa seeds would not be impacted by the naturally occurring commensal bacterial species present on untreated alfalfa seeds. Several processes such as acidic electrolyzed water, cold plasma, high pressure processing, hot water,

ultrasound, pulsed light, ultraviolet irradiation, and gamma irradiation have been used to effectively decontaminate the seed surface (Mir et al., 2021; Puligundla et al., 2017; Wuytack et al., 2003). However, chemical treatments using compounds such as chlorine, acetic acid, or calcium hypochlorite are commonly used to decontaminate the surface of sprouting seeds (Mir et al., 2021). Using a protocol developed by Wang & Kniel, a three stage seed surface decontamination protocol was developed that used a 70% ethanol treatment, followed by a 10% bleach treatment, and a final distilled water rinse (Wang & Kniel, 2014). However, this protocol was expanded upon by adding the following: an initial rinse using distilled water and Tween-20 to remove loosely bound particles from the seeds, an increase in ethanol concentration from 70% to 95%, and the use of a variable amount of bleach ranging from 5% to 20%. Additionally, ISS and FTL alfalfa seeds were tested under the various bleach conditions to observe potential differences in the ability of the seeds' commensal microorganisms to survive the seed surface decontamination process (Figure 2).

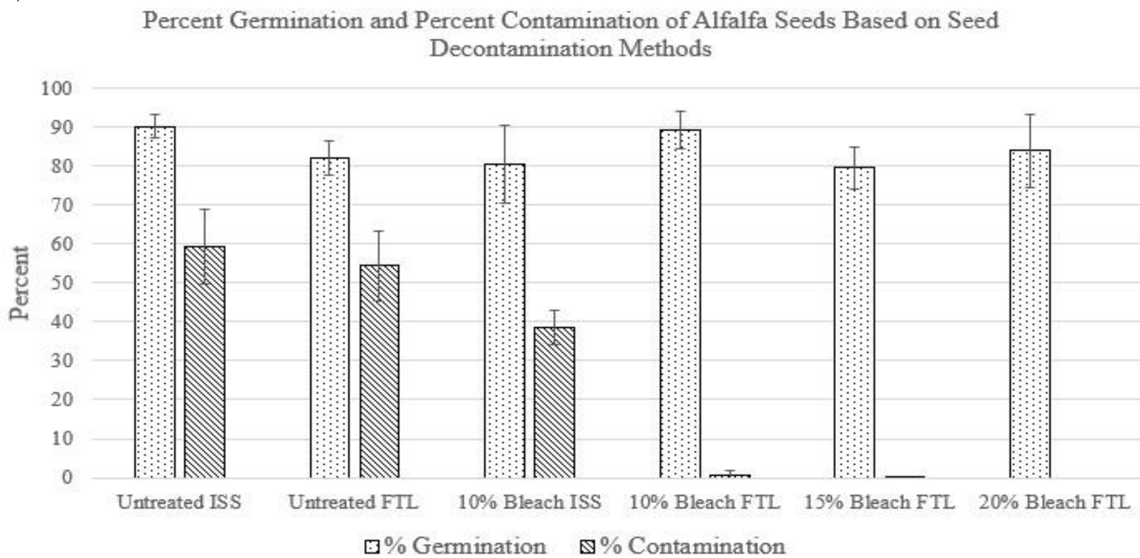

Figure 2: Percent germination and percent contamination of either ISS or FTL alfalfa seeds based on a varying percentage of commercial bleach used in the seed decontamination process. n = 3 for all treatments. Error bars represent sample standard deviation.

Based on Figure 2, the percentage of untreated seeds that were contaminated with a commensal bacterial species capable of growing on R2A agar at 25°C was higher in ISS seeds (59% ± 9.5%) compared to FTL seeds (55% ± 8.9%) (Figure 2). However, the percent germination of the FTL seeds (82% ± 4.5%) was lower compared to the ISS seeds (90% ± 3.0%) (Figure 2). When comparing the 10% commercial bleach treatments, the percent contamination of the FTL seeds (0.83% ± 1.2%) was lower than that of the ISS seeds (38.4 ± 9.8%) (Figure 2). Therefore, the FTL seeds were selected for further testing using higher commercial bleach concentrations. As a result, FTL seeds that were treated with a 20% commercial bleach solution were found to be completely decontaminated of surface bacterial species without impacting the percent germination of the FTL seeds

(Figure 2). Based on these results, FTL seeds were selected to be used for all further alfalfa-related experiments and the seed surface decontamination protocol was adjusted to use 20% commercial bleach.

2.3.2 Isolation, Characterization, and Identification of Plant-Associated Bacteria

Plant-associated bacteria (PAB) that could be isolated from alfalfa seeds or sprouts were selected for potential biocontrol agents due to two factors. The first factor being that previous work has found that bacterial genera such as *Pseudomonas, Pantoea, Serratia, Enterobacter, Hafnia, Erwinia,* and *Bacillus* have had a variable impact on *Salmonella in planta* that resulted in 1 to 8 log reductions (Fett, 2006; Kim et al., 2020; Oliveira et al., 2015; Shen et al., 2017). The second factor that influenced the decision to isolate PAB as biocontrol agents was because many of these microorganisms are considered plant-growth promoting bacteria. These species are known to have a mutualistic relationship with the host plant through the production of plant growth stimulating hormones, the fixation of nitrogen, and the facilitation of nutrient acquisition via organic acids or siderophores (Morales-Cedeno et al., 2021). Furthermore, López et al. have isolated and identified 40 different bacterial genera from alfalfa seeds indicating that the microbiota of alfalfa is diverse and biocontrol agent isolation has great potential (López et al., 2018). To isolate PAB from ISS and FTL alfalfa seeds, 10 gram aliquots of surface decontaminated and untreated alfalfa seeds were sprouted; samples were collected on the 0, 1, 3, and 6 day time points; the PAB were extracted from the plant tissue; and the PAB were enumerated (Figures 3 and 4).

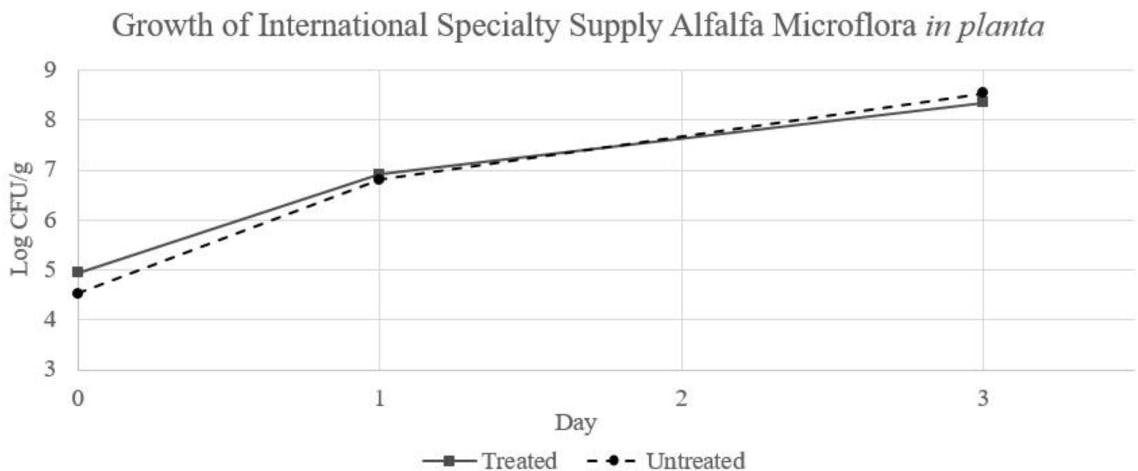

Figure 3: The cell density of plant-associated bacteria extracted and enumerated from treated (surface decontaminated) and untreated International Specialty Supply alfalfa seeds over a three day period of time. n = 1 for all samples.

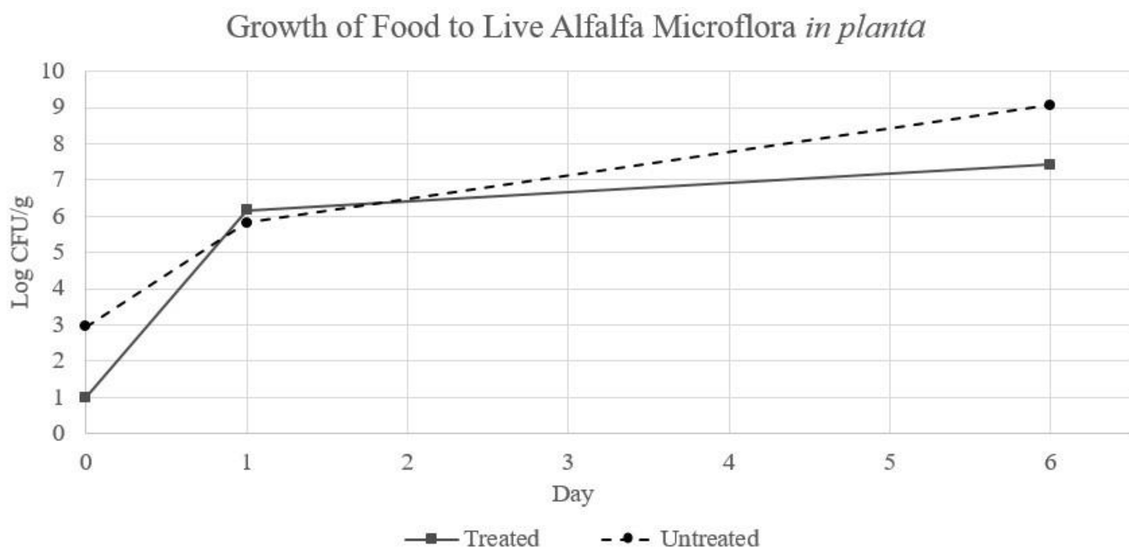

Figure 4: The cell density of plant-associated bacteria extracted and enumerated from treated (surface decontaminated) and untreated Food to Live alfalfa seeds over a six day period of time. n = 1 for all samples.

Based on Figure 3, there was a slight decrease in the initial PAB cell density of the ISS seeds when comparing the untreated (5 log CFU/g) to the treated (4.5 log CFU/g) alfalfa seeds indicating that the seed surface decontamination protocol developed in the aforementioned was not effective in decontaminating the ISS seed surface (Figure 3). Furthermore, the initial PAB cell density of the FTL treated seeds (1 log CFU/g) was lower compared to the untreated seeds (3 log CFU/g) (Figure 3). Therefore, this result highlights the inefficiencies of seed surface decontamination methodologies and the potential impact on future experiments using the FTL seeds. The PAB cell density of the treated and untreated ISS sprouts increased exponentially within 24 hours to 7 log CFU/g and increased further to 8.5 log CFU/g after 3 days of seed sprouting (Figure 3). A similar result was observed in the FTL sprouts where the PAB cell densities increased exponentially in both seed samples to 6 log CFU/g within 24 hours and the cell density increased further to 7.4 and 9 log CFU/g for the treated and untreated seeds, respectively (Figure 4). Since the sprouts were grown on 1% water-agar, a minimal medium, there is strong evidence indicating that the exponential growth of the PAB was being supported by exudates secreted from the alfalfa sprouts.

Although the enumeration of the PAB was important to understanding the growth kinetics of alfalfa seeds' commensal microbial community, 28 PAB isolates extracted from the ISS treated seeds, ISS untreated seeds, FTL treated seeds, and FTL untreated seeds were picked, streaked for single colonies on LB agar, and characterized. In addition to the isolates obtained, Raghav Garg, an undergraduate researcher, isolated an additional 18 PAB from store-bought alfalfa sprouts that were also characterized. The PAB isolates were characterized based on three methods: colony morphology, Gram stain/cell morphology, and ability to grow on XLT4 media (Table 5). XLT4 was used during the characterization

process to screen for potential *Salmonella* isolates and *Salmonella* isolates were identified based on the formation of black colonies due to the reduction of hydrogen sulfide to form a black precipitate (Miller et al., 1991).

Table 5: The qualitative characteristics of the plant-associated bacterial isolates regarding colony morphology on LB, Gram stain, cell morphology, and ability to grow on XLT4 agar. Isolates were obtained from three sources: Food to Live (FTL) alfalfa seeds/sprouts, International Specialty Supply (ISS) alfalfa seeds/sprouts, and store-bought alfalfa sprouts (SBAS).

PAB Isolate	Source of Isolate	Colony Morphology	Gram Stain & Cell Morphology	Growth on XLT4
JV1	FTL	Circular, flat, smooth, mucoid colony with white coloration.	Gram positive, large rod-shaped cells.	None
JV2	FTL	Irregular, flat, undulated colony with white coloration. Colony appears rough and wrinkled.	Gram positive, large rod-shaped cells.	None
JV3	FTL	Circular, flat, smooth colony with white, opaque coloration. Colony appears moist.	Gram negative, small rod-shaped cells.	White colonies with black precipitate in the colony center.
JV4	FTL	Irregular, flat colony with white coloration. Swarming observed.	Gram positive, large rod-shaped cells.	None
JV5	FTL	Circular, flat, smooth colony with yellow, translucent coloration. Colony appears moist.	Gram negative, small rod-shaped cells.	None
JV6	FTL	Circular, flat, smooth colony with yellow, translucent coloration. Colony appears moist.	Gram negative, small rod-shaped cells.	None
JV7	FTL	Circular, flat, undulated colony with white, opaque coloration. Colony appears dry.	Gram positive, very large rod-shaped cells.	None

PAB Isolate	Source of Isolate	Colony Morphology	Gram Stain & Cell Morphology	Growth on XLT4
JV8	FTL	Circular, flat, undulated colony with white, opaque coloration. Colony appears dry.	Gram positive, very large rod-shaped cells.	None
JV9	FTL	Circular, flat, undulated colony with white, opaque coloration. Colony appears dry.	Gram positive, very large rod-shaped cells.	None
JV10	FTL	Irregular, flat, lobated colony with white opaque/translucent coloration. Colony appears mucoid. Swarming observed.	Gram negative, medium rod-shaped cells.	None
JV11	FTL	Irregular, flat, lobated colony with white, opaque coloration. Colony appears mucoid.	Gram positive, medium rod-shaped cells.	None
JV12	FTL	Filamentous, flat, undulated, colony with white opaque/translucent coloration.	Gram positive, medium rod-shaped cells.	None
JV13	FTL	Circular, flat colony with rough center and smooth exterior. White, opaque coloration. Colony appears moist on the smooth exterior.	Gram positive, small rod-shaped cells.	None
JV14	FTL	Circular, flat colony with rough center and smooth exterior. White, opaque coloration. Colony appears moist on the smooth exterior.	Gram negative, small rod-shaped cells.	None
JV15	ISS	Punctiform, flat, smooth colony with yellow, opaque coloration. Colony appears moist.	Gram negative, very small rod-shaped cells.	None

PAB Isolate	Source of Isolate	Colony Morphology	Gram Stain & Cell Morphology	Growth on XLT4
JV16	ISS	Circular, flat, smooth colony with white, opaque color.	Gram negative, very small rod-shaped cells.	None
JV17	ISS	Circular, flat, smooth colony with white, opaque coloration.	Gram negative, very small rod-shaped cells.	None
JV18	ISS	Circular, flat, smooth colony with yellow-white, opaque coloration.	Gram negative, very small rod-shaped cells.	None
JV19	ISS	Circular, smooth, flat with yellow-white, opaque coloration.	Gram negative, very small rod-shaped cells.	Pink colonies
JV20	ISS	Circular, smooth, flat with yellow-white, opaque coloration.	Gram negative, very small rod-shaped cells.	Pink colonies
JV21	ISS	Circular, smooth, flat with yellow-white, opaque coloration.	Gram negative, very small rod-shaped cells.	Pink colonies
JV22	ISS	Circular, smooth, flat with yellow-white, opaque coloration.	Gram negative, very small rod-shaped cells.	Pink colonies
JV23	ISS	Circular, smooth, flat with yellow, opaque coloration.	Gram negative, small rod-shaped cells.	None
JV24	ISS	Circular, undulated, flat with white, opaque coloration.	Gram positive, very large rod-shaped cells	None
JV25	ISS	Circular, smooth, flat with yellow, opaque coloration.	Gram negative, small rod-shaped cells.	None
JV26	ISS	Circular, smooth, flat with yellow-white, opaque coloration.	Gram negative, medium rod-shaped cells	None

PAB Isolate	Source of Isolate	Colony Morphology	Gram Stain & Cell Morphology	Growth on XLT4
JV27	ISS	Circular, smooth, flat with yellow-white, opaque coloration.	Gram negative, medium rod-shaped cells	Pink colonies
JV28	ISS	Circular, smooth, flat with yellow-white, opaque coloration.	Gram negative, medium rod-shaped cells	None
RG1	SBAS	Circular, smooth flat with white, opaque coloration.	Gram negative, small rod-shaped cells	None
RG2	SBAS	Punctiform, smooth, flat with white, opaque coloration.	Gram negative, small rod-shaped cells	Pink colonies
RG3	SBAS	Circular, smooth, flat with white, opaque coloration.	Gram negative, very small rod-shaped cells	Pink colonies
RG4	SBAS	Circular, smooth, flat with white, opaque coloration.	Gram negative, small rod-shaped cells	None
RG5	SBAS	Circular, smooth, flat with yellow-white, opaque coloration.	Gram negative, small rod-shaped cells	None
RG6	SBAS	Circular, smooth, flat colony with wrinkled edges and smooth white colony center.	Gram negative, small rod-shaped cells	None
RG7	SBAS	Circular, smooth, flat with yellow-white, opaque coloration.	Gram negative, very small rod-shaped cells	None
RG8	SBAS	Circular, smooth, flat with white, opaque coloration	Gram negative, small rod-shaped cells	White colonies and yellowing of the media
RG9	SBAS	Circular, smooth, flat with yellow-white, opaque coloration.	Gram negative, small rod-shaped cells	None

PAB Isolate	Source of Isolate	Colony Morphology	Gram Stain & Cell Morphology	Growth on XLT4
RG10	SBAS	Circular, smooth, flat with white coloration. Colonies appear slightly mucoid.	Gram negative, very small rod-shaped cells	None
RG11	SBAS	Circular, smooth, flat with yellow-white, opaque coloration.	Gram negative, very small rod-shaped cells	None
RG12	SBAS	Circular, smooth, flat with yellow-white, opaque coloration.	Gram negative, very small rod-shaped cells	None
RG13	SBAS	Circular, smooth, flat with white, opaque coloration.	Gram negative, medium rod-shaped cells	None
RG14	SBAS	Circular, smooth, flat with yellow-white, opaque coloration.	Gram negative, small rod-shaped cells	None
RG15	SBAS	Circular, smooth, flat with yellow-white coloration. Colonies are mucoid.	Gram negative, medium rod-shaped cells	None
RG16	SBAS	Punctiform, smooth, flat with white opaque coloration.	Gram negative, medium rod-shaped cells	None
RG17	SBAS	Circular, undulated, flat colony with wrinkled edge and white colony center.	Gram negative, small rod-shaped cells	None
RG18	SBAS	Circular, smooth, flat with yellow-white, opaque coloration. Colonies appear slightly mucoid.	Gram negative, very small rod-shaped cells	None

When comparing the general morphological characteristics of the 42 PAB isolates, there was a wide variety in the shape and coloration of each isolate. With regards to color, half of the isolates were a white opaque coloration and the other half were a yellow or yellow-white color (Table 5). However, the greatest variation was observed in the PAB isolates' morphological characteristics. Isolates such as JV2, JV4, JV7-JV12, and JV24 appeared as either filamentous, undulated, or lobated; furthermore, the isolates had either a rough, dry appearance or a mucoid, moist appearance (Table 5). Of these nine isolates, JV4 and JV10 appeared to display swarming motility where a translucent halo of bacterial growth emanating from a single colony allowed these isolates to completely envelop the LB plate (Table 5). Swarming motility is a process utilized by bacterial genera such as *Proteus, Vibrio, Bacillus,* and *Clostridium* where vegetative cells will differentiate into hyper-flagellated cells (Fraser & Hughes, 1999). With this hyper-flagellation, the cells align to form rafts that can move via a coordinated flagellar movement away from the original vegetative population (Fraser & Hughes, 1999). Furthermore, swarming motility has been found to be a contributing factor towards root colonization by the biocontrol agent *Bacillus subtilis* SWR01 (Gao et al., 2016). Therefore, JV4 and JV10 may be ideal biocontrol agents since swarming motility could allow these isolates to rapidly colonize the surface of alfalfa and outcompete *Salmonella* for physical space.

Following a Gram stain and visualization under a microscope, 10 isolates were identified as Gram positive and 32 isolates were identified as Gram negative (Table 5). All of the isolates were rod-shaped; however, the relative size varied drastically with isolates such as JV7-JV9 and JV24 being the largest cells compared to the smallest isolates, JV3, JV6, and RG18 (Table 5). Lastly, each isolate was streaked onto XLT4 agar to screen for potential *Salmonella* isolates and to identify other members of Enterobacteriaceae (Table 5). Amongst the isolates, JV19-JV22, JV27, and RG2-RG3 grew to form pink colonies and RG8 grew to form white colonies that changed the media color to yellow (Table 5). Based on these results, these isolates were not identified as *Salmonella*; however, these isolates could be other members of Enterobacteriaceae such as *Klebsiella, Enterobacter*, *Citrobacter*, or *Escherichia coli* (Miller et al., 1991). In contrast, JV3 grew to form white colonies with a black colony center (Table 5). According to Miller et al., *Salmonella* isolates create a black precipitate on XLT4 due to the reduction of H_2S; therefore, JV3 was recorded as a presumptive *Salmonella* isolate for further identification (Miller et al., 1991).

Although the morphological characteristics of the PAB isolates and their reaction on XLT4 can provide a general picture of their identity, these initial qualitative characteristics did not provide a clear idea of each isolate's identity. Therefore, the 16S rRNA gene of each PAB isolate was amplified and submitted for Sanger sequencing. After sequencing, 16 of the 48 PAB isolates were identified to either the genus, species, or strain level (Table 6).

Table 6: The identification of 16 plant-associated bacterial isolates extracted from three different alfalfa sources. Isolates were identified via the 16S rRNA gene.

PAB Isolate	Identification	PAB Isolate	Identification
JV3	*Salmonella enterica* Enteritidis	RG1	*Pseudomonas sp.* #1
JV5	*Pantoea sp.* #1	RG3	*Raoultella terrigena*
JV6	*Pantoea agglomerans*	RG7	*Enterobacter ludwigii*
JV7	*Priestia megaterium* #1	RG9	*Paenibacillus sp.* #1
JV8	*Priestia megaterium* #2	RG14	*Pantoea sp.* #2
JV13	*Bacillus sp.* #1	RG15	*Paenibacillus sp.* #2
JV22	*Enterobacter sp.* #1	RG17	*Pseudomonas rhodesiae*
JV24	*Priestia megaterium* #3	RG18	*Pantoea sp.* #3

Based on the 16S rRNA sequencing results, the presumptive *Salmonella* isolate characterized as JV3 was identified as *Salmonella enterica* Enteritidis (Table 6). Although this particular serotype of *Salmonella* is commonly associated with animal sources such as reptiles, chickens, and undercooked eggs; seven outbreaks of *Salmonella* Enteritidis occurred in mung bean sprouts between 2000 and 2002 (Mohle-Boetani et al., 2009). Furthermore, an outbreak of *Salmonella* Enteritidis that occurred in 2011 was linked to alfalfa sprouts which further confirms that *Salmonella* Enteritidis can be associated with sprouts and/or sprouting seeds (CDC: Alfalfa, 2011). Therefore, the identification of JV3 as *Salmonella* Enteritidis from alfalfa sprouting seeds does align with this strain's host range. However, this finding further supports the saliency of *Salmonella* contamination in sprouting seeds since JV3 was isolated from the Food to Live sprouting seeds that were purchased online from Amazon.com. As a result, there is evidence that contaminated lots of alfalfa sprouting seeds are available on the market for the average consumer to purchase and germinate at home; therefore, consumers could be at risk of growing/consuming *Salmonella*-contaminated sprouts without knowing of the hidden risk.

In addition to confirming the presumptive *Salmonella* isolate, the 16S rRNA sequencing identified three of the eight isolates that grew on XLT4 without producing a black precipitate as members of either *Enterococcus* or *Raoultella*, a close relative of *Klebsiella* (Drancourt et al., 2001) (Table 6). Therefore, the 16S rRNA sequencing identification further confirms that the non-*Salmonella* isolates that grew on XLT4 are indeed members of Enterobacteriaceae. However, these isolates were not selected for

further biocontrol agent analysis because XLT4 was the main selective and differential media used for downstream biocontrol experiments.

With regards to potential biocontrol agents that were identified, numerous PAB isolates were identified as *Pantoea sp.* and one isolate was identified as *Pantoea agglomerans*. The genus *Pantoea* is a Gram negative, rod-shaped member of the Enterobacteriaceae family and *Pantoea* colonies are identified based on their yellow pigmentation (Walterson & Stavrinides, 2015). Additionally, *Pantoea spp.* are known plant-associated bacteria and these bacteria have been isolated from store-bought alfalfa sprouts (López et al., 2018). Based on this information, twenty PAB isolates' morphological characteristics align with *Pantoea*; however, only four isolates were successfully identified as *Pantoea sp*. (Table 5). Furthermore, one isolate, JV6, was identified as *Panotea agglomerans* (Table 6). *Pantoea agglomerans* is a known opportunistic pathogen of animals, plants, and humans; however, the species' pathogenicity varies based on the presence of plasmids containing pathogenicity islands that encode for virulence factors such as type III secretion systems (Dutkiewicz et al.: Part III, 2016). For *Pantoea agglomerans* strains that lack pathogenicity islands, *Pantoea agglomerans* has been found to be an effective plant-growth promoting species through either nitrogen fixation or the production of phytohormones (Dutkiewicz et al.: Part IV, 2016). Furthermore, *Pantoea agglomerans* is a known biocontrol agent of bacterial and fungal plant pathogens making *Pantoea agglomerans* a potential candidate for biocontrol of *Salmonella* (Dutkiewicz et al.: Part IV, 2016). As a result, two strains of *Pantoea agglomerans* (P10c and E325) have been accepted for agricultural use in the United States and New Zealand (Guevarra et al., 2021). In addition to *Pantoea*, the remaining Gram-negative PAB isolates were identified as either *Pseudomonas sp.* or *Pseudomonas rhodesiae* (Table 6). Similar to *Pantoea*, *Pseudomonas* has been isolated and identified from store-bought alfalfa sprouts implying that *Pseudomonas spp.* have a natural presence on alfalfa (López et al., 2018). *Pseudomonas spp.* such as *P. fluorescens* have displayed promising biocontrol abilities in regulating both human and plant pathogens (Fett, 2006; Quagliotto et al., 2009). Furthermore, *Pseudomonas rhodesiae* has been found to display plant growth promoting benefits and cause the induction of systemic acquired resistance systems in inoculated pepper plants (Kang et al., 2007).

Following *Pantoea* and *Pseudomonas*, the next grouping of isolates that were identified were members of *Bacillus*, *Priestia megaterium*, and *Paenibacillus* (Table 6). As previously mentioned, *Bacillus spp.* provide great potential for biocontrol due to the species natural presence in alfalfa and previous work that has shown that an endophytic strain of *B. subtilis* was capable of reducing internalized *Salmonella* 1 to 1.5 logs after 5 days of incubation (López et al., 2018; Shen et al., 2017). Similar to *Bacillus*, *P. megaterium* has been previously isolated from the rhizosphere of alfalfa and these isolates were found to have a plant-growth promoting effect through actions such as siderophore production, Nitrogen fixation, phosphate solubilization, and phytohormone production

(Flores-Duarate et al., 2022). Furthermore, *P. megaterium* has been used to effectively combat plant diseases such as brown root rot, damping-off, and soybean cyst nematode infections due to the species' ability to produce proteases, chitinases, and peroxidases (Chakraborty et al., 2006; Solanki et al., 2012; Zhou et al., 2017). The final *Bacillus*-like isolates that were identified as *Paenibacillus* were different from isolates of *Bacillus* and *P. megaterium* because the *Paenibacillus* isolates were identified as Gram negative (Table 5). However, this particular genus is interesting in that *Paenibacillus* cells can be stained as Gram positive, Gram negative, or Gram variable; therefore, the morphological characteristics remain true to the 16s RNA sequence identification (Grady et al., 2016). Similar to *Bacillus* and *Priestia*, *Paenibacillus spp.* are a natural commensal endophytic microorganism of alfalfa (Lai et al., 2015; López et al., 2018). Additionally, *Paenibacillus spp.* have been considered for biocontrol due to their ability to produce a wide array of antimicrobial compounds such as polymyxins (Rybakova et al., 2016).

2.3.3 Plant-Associated Bacteria Inoculation

Potential biocontrol agents were assessed based on two factors: a positive influence on biomass production and the ability to attain a high cell density *in planta*. PAB isolates that displayed a positive effect on alfalfa biomass development could be through the production of growth stimulating hormones such as auxins (López et al., 2018; Morales-Cedeno et al., 2021). Due to this pre-existing commensal relationship with the alfalfa sprout, plant-associated bacterial species have been found to reach high cell densities upwards of 10^9 CFU/g of plant tissue (Gnanamanickam, 2006). In the context of biocontrol, *Salmonella* would be controlled via competitive exclusion where the plant-associated bacteria exist at their peak cell density to limit the amount of space on the sprout surface and/or consume limiting exudates produced by the sprout (Fett, 2006; Kim et al., 2020; Matos & Garland, 2005). To assay the PAB isolates for the aforementioned biocontrol agent criteria, 14 PAB isolates were selected based on differences in their phenotypic characteristics outlined in Table 5 and their identity outlined in Table 6, then 2.5 gram aliquots of alfalfa seeds were inoculated with a single PAB isolate. Additionally, *Pseudomonas putida* KT-2440 was assayed because *P. putida* KT-2440 is a known plant-associated bacterial species and this strain of *P. putida* can be genetically modified to express foreign genes that may enhance its ability as a biocontrol agent (Wu et al., 2011).

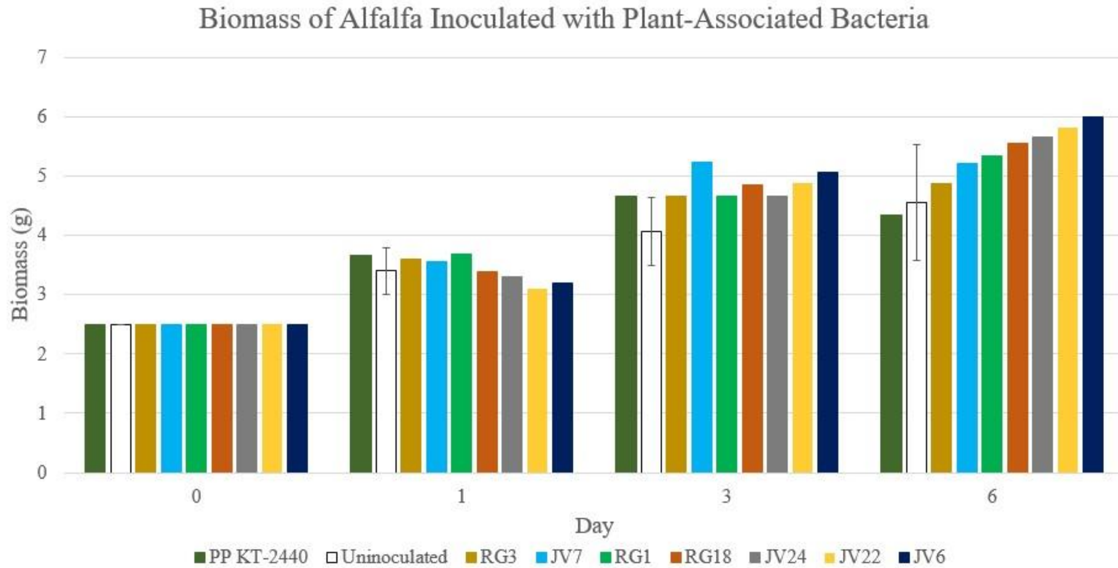

Figure 5: The development of alfalfa biomass in response to a 4.5 to 5 log CFU/g inoculation of plant-associated bacteria and subsequent incubation for a 6 day period of time. n = 5 for uninoculated alfalfa seeds/sprouts and n = 1 for inoculated alfalfa seeds/sprout samples. Error bars represent sample standard deviation. PP KT-2440, RG3, JV7, RG1, RG18, JV24, JV22, and JV6 correspond to *Pseudomonas putida* KT-2440, *Raoultella terrigena*, *Priestia megaterium* #1, *Pseudomonas sp.* #1, *Pantoea sp.* #3, *Priestia megaterium* #3, *Enterobacter sp.* #1, and *Pantoea agglomerans*, respectively.

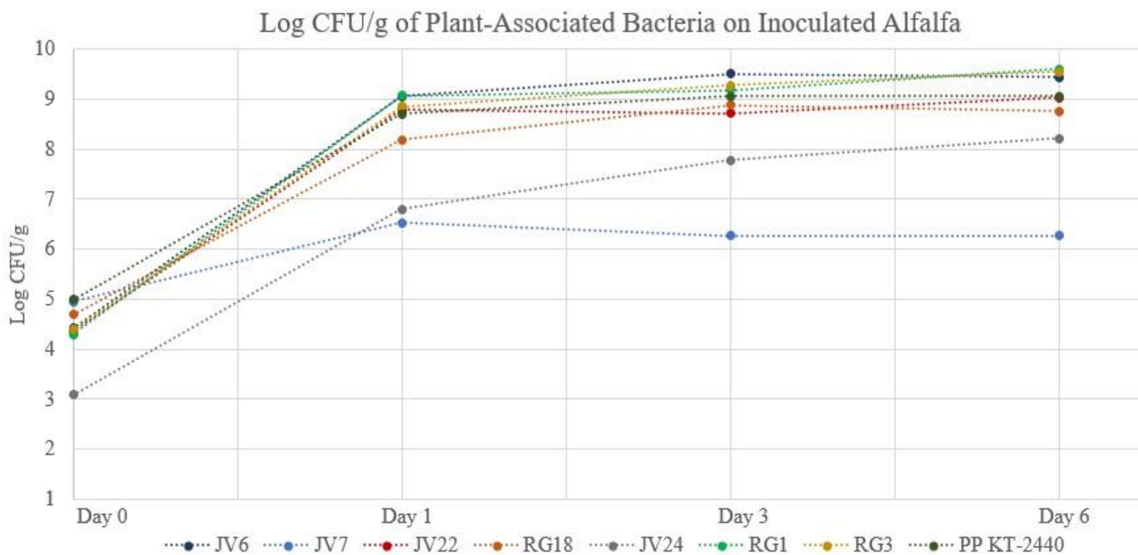

Figure 6: The development of plant-associated bacterial cell density *in planta* following a 3 to 5 log CFU/g inoculation and subsequent incubation for a 6 day period of time. n = 1 for all samples. PP KT-2440, RG3, JV7, RG1, RG18, JV24, JV22, and JV6 correspond to *Pseudomonas putida* KT-2440, *Raoultella terrigena*, *Priestia megaterium* #1, *Pseudomonas sp.* #1, *Pantoea sp.* #3, *Priestia megaterium* #3, *Enterobacter sp.* #1, and *Pantoea agglomerans*, respectively.

The impact that each PAB isolate had on alfalfa biomass development was recorded over a 6 day period (Figure 6). Of the 8 PAB isolates that had an intermediate to strong beneficial impact on biomass development, RG18, JV24, JV22, and JV6 exceeded the error observed in the uninoculated alfalfa sprout biomass (Figure 6). The isolates RG1, RG3, and JV7 had a beneficial impact on biomass production; however, the final biomass did not exceed the error observed in the uninoculated alfalfa sprout biomass (Figure 6). Finally, *P. putida* KT-2440 was found to have a slightly detrimental effect on alfalfa biomass production that was within the error observed in the uninoculated alfalfa sprout biomass (Figure 6).

Regarding the progression of the PAB cell densities over the sprout germination period, JV22, RG18, JV6, RG1, RG3, and *P. putida* KT-2440 displayed a great amount of growth from an inoculum of 4.3 to 5 log CFU/g to 8.8 to 9 log CFU/g within 24 hours of germination (Figure 7). Over the next 5 days of germination, the cell densities for these isolates increased further and stabilized at 8.75 to 9.6 log CFU/g (Figure 7). Of the 8 PAB isolates, both JV24 and JV7 had a slower progression of cell density in the inoculated alfalfa (Figure 7). However, JV24 was inoculated at 3 log CFU/g, roughly 2 logs lower than JV7, and JV24's final cell density was 8.2 log CFU/g after 6 days of germination (Figure 7). Therefore, JV24 was found to thrive on alfalfa sprouts. In comparison, JV7 was inoculated at 5 log CFU/g and the final cell density only increased to 6.3 log CFU/g indicating that JV7 was not well adapted to growing to a high cell density on alfalfa sprouts (Figure 7).

Therefore, a cocktail of plant-associated bacteria was assembled based on the PAB isolates that displayed the greatest beneficial impact on alfalfa biomass development and also attained a final cell density of 8 log CFU/g or higher to effectively outcompete *Salmonella* for space and limiting exudates on alfalfa sprouts. The number of isolates in the cocktail was limited to a total of 5 isolates to maintain the cocktail's simplicity for further experimentation. Based on the two criteria assessed, JV6, JV24, RG1, RG18, and *P. putida* KT-2440 were selected to be used for the biocontrol agent cocktail. JV22 was a prospective candidate for selection; however, JV22 was found to grow on XLT4 agar which was the main method to differentiate between PAB isolates and *Salmonella* (Table 5). Therefore, JV22 could not be used for further experimentation. Although *P. putida* KT-2440 did not have a significant impact on the development of alfalfa biomass, *P. putida* KT-2440 was included in the PAB cocktail due its ability to attain a high cell density on alfalfa and the potential to genetically modify this organism to enhance its plant growth promoting effects and/or biocontrol potential.

2.3.4 Biocontrol Agent Cocktail: Biomass Development, Shelf-Life Study, and Whole Genome Sequencing

Once a biocontrol agent cocktail was developed, the cocktail was assayed against three criteria: the PAB cocktail stimulated alfalfa biomass development, the PAB cocktail achieved a high cell density without compromising the diversity of the cocktail, and the

shelf-life of the inoculated alfalfa sprouts were comparable to uninoculated sprouts. Therefore, eight 5 gram samples of FTL alfalfa sprouting seeds were inoculated with 7 log CFU/g of the PAB cocktail outlined in Table 4, then the development of alfalfa biomass and PAB cell density was monitored with respect to time (Figures 8 and 9).

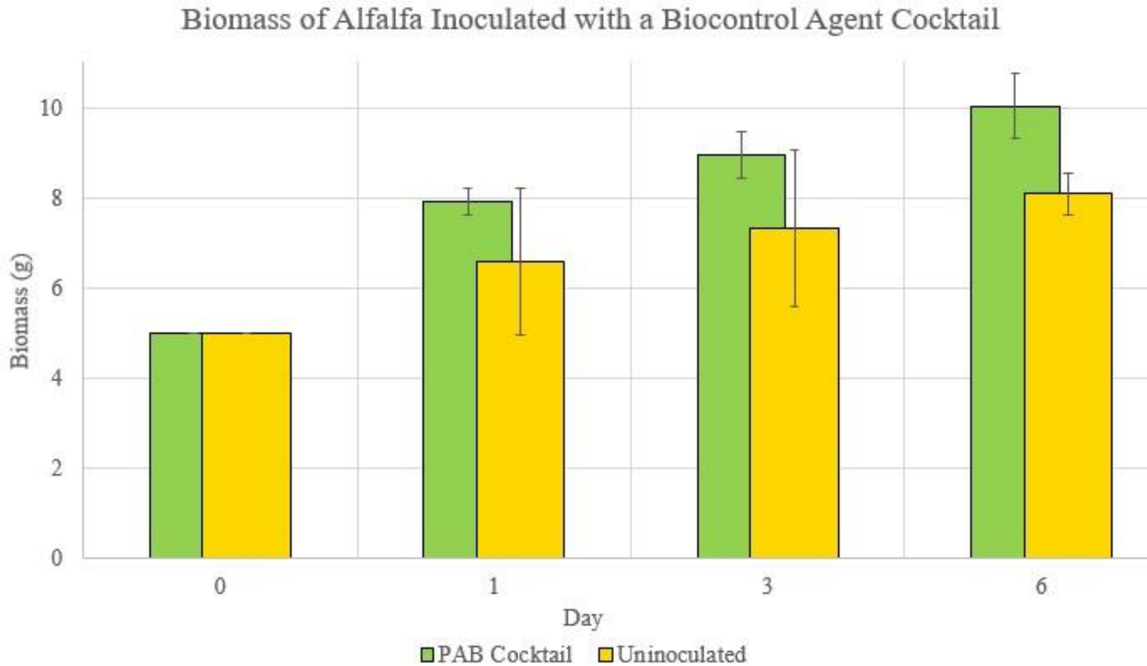

Figure 7: The development of alfalfa biomass in response to a 7 log CFU/g inoculation of a plant-associated bacteria cocktail and subsequent incubation for a 6 day period of time. n = 3 for uninoculated alfalfa seeds/sprouts and n = 8 for alfalfa seeds/sprouts inoculated with the PAB cocktail. Error bars represent sample standard deviation.

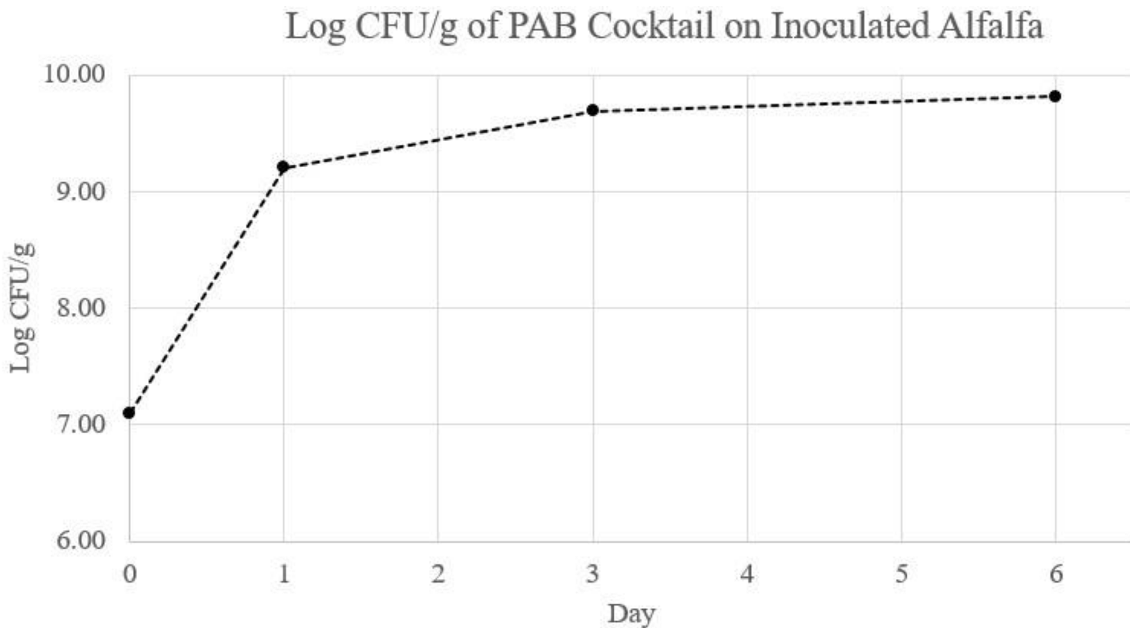

Figure 8: The development of plant-associated bacterial cell density *in planta* following a 7 log CFU/g inoculation and subsequent incubation for a 6 day period of time. A single replicate was performed to verify previous results.

Based on Figure 8, the PAB cocktail had a beneficial effect on alfalfa biomass development by day 6 of germination (Figure 7). Furthermore, seeds that were inoculated with the PAB cocktail displayed elevated biomass production compared to the uninoculated control on day 1 and 3, but these results were within error compared to the uninoculated control (Figure 7). In terms of final alfalfa biomass development, the PAB cocktail sample was on average 25% higher by the sixth day of germination (10 grams) compared to the average biomass of the uninoculated alfalfa sprouts (8 grams) (Figure 7).

Therefore, the PAB cocktail has great potential in a plant-growth promoting capacity; however, this effect may be limited to a shorter germination period as indicated by the plateauing observed for the PAB cocktail samples. With respect to the development of PAB cell density, the PAB cocktail was inoculated at 7 log CFU/g and the cell density increased to 9.2 log CFU/g within the first 24 hours of germination (Figure 8). The PAB cell density continued to increase during germination and the final cell density was 9.8 log CFU/g (Figure 8). Based on this result, the PAB cocktail appeared to be consistent with previous data discussed in Figure 7 confirming that the PAB cocktail's constituents were capable of thriving on alfalfa sprouts. On the sixth day of germination, a picture was taken of the LB plate used to enumerate the PAB cocktail constituents isolated from the inoculated alfalfa sprouts, and this picture was used to determine the relative abundance of each constituent (Figure 9).

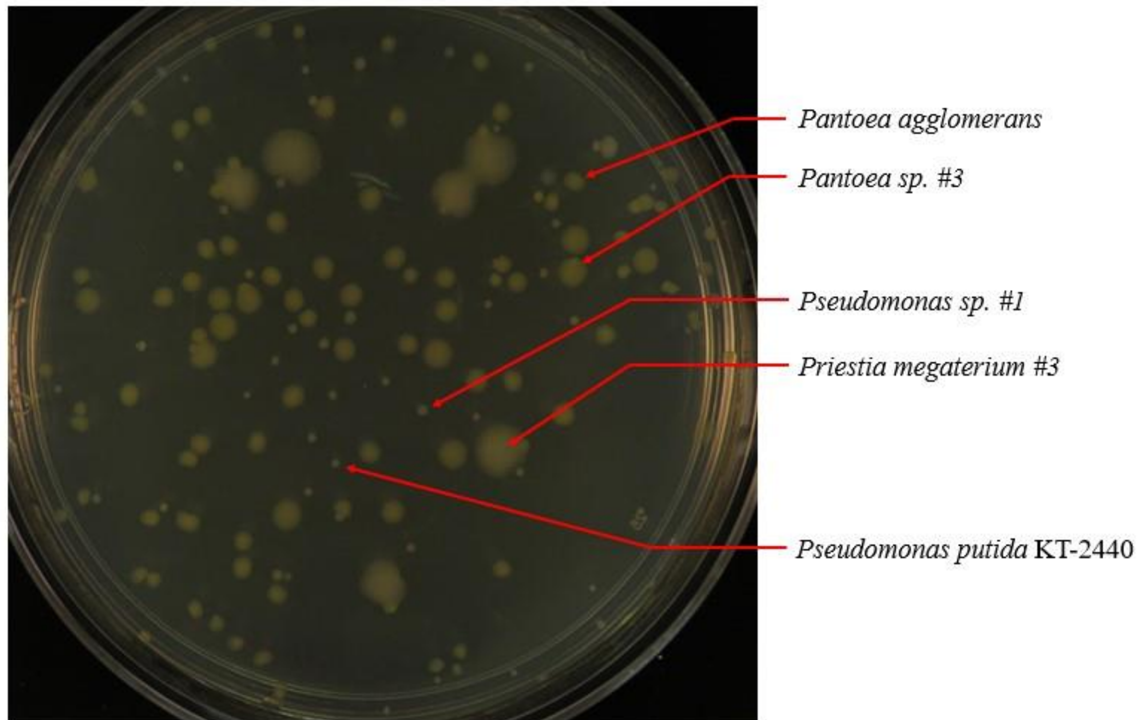

Figure 9: A photograph of an LB plate that indicates each PAB cocktail constituent and reflects the abundance of each PAB cocktail constituent extracted from alfalfa sprouts following six days of germination. The LB plate reflects the 10^{-6} dilution of the day six PAB extract.

An estimation of the relative abundance of each PAB cocktail constituent was determined to be 31.3%, 25.7%, 19.4%, 19.4%, and 4.2% for *Pseudomonas sp. #1, Pantoea agglomerans, Pantoea sp. #3, Pseudomonas putida* KT-2440, and *P. megaterium #3*, respectively (Figure 9). Of the five PAB cocktail constituents, *Pantoea* and *Pseudomonas* constituents were the predominant members of the microbial community that were observed at the 10^{-6} dilution, and each isolate was identified in relatively equal proportions (Figure 9). In contrast, the *P. megaterium* constituent was in the minority; however, *P. megaterium* was still observed at the 10^{-6} dilution, indicating that the constituent's cell density had not drastically faltered in comparison to other members of the PAB cocktail (Figure 10). Therefore, there was no prevalent evidence that a single member of the PAB cocktail showed antagonistic effects towards each other based on each member's relative abundances. The PAB cocktail was then further assessed for antagonistic effects towards alfalfa sprouts by conducting a shelf-life study in which inoculated alfalfa sprouts were compared to uninoculated alfalfa sprouts over a sixteen day time period (Table 7).

Table 7: Qualitative observations regarding the shelf life of surface decontaminated Food to Live alfalfa seeds that have been either treated with a biocontrol agent cocktail (7.1 log CFU/g initial inoculum) or left untreated (Control). Day 1 of the shelf life study started 6 days after initial seed germination.

Day Post-Harvest	Biocontrol Agent Cocktail	Control #1	Control #2	Control #3
1	Large, thick sprouts with brown tips. Sprouts appear firm.	Small, thin sprouts with no browning. Sprouts appear firm.	A mix of thick and thin sprouts of varying sizes. No browning observed. Sprouts appear firm. Mold growth observed during harvest.	A mix of thick and thin sprouts of varying sizes. No browning observed. Sprouts appear firm. Mold growth observed during harvest.
4	Sprouts have retained size and rigidity. Browning of sprout tips has not spread.	Sprouts have retained size and rigidity. Browning of sprout tips observed.	Sprouts have retained size and rigidity. Mold growth observed.	Sprouts have retained size and rigidity.

7	No change	No change	No change	No change
10	No change	No change	No change	No change
13	No change	No change	Mold growth spreading.	Mold growth observed
16	No change	No change	No change. Mold growth was not spreading further.	No change. Mold growth was not spreading further.

According to Keshri et al., the shelf-life of alfalfa sprouts stored under refrigerated conditions is approximately 3 weeks; therefore, the 16 day shelf-life study that was conducted was slightly shorter than previous studies (Keshri et al., 2019). Over the course of this shelf-life study, the sprouts were assessed for attributes such as browning, loss of rigidity/firmness, and presence of mold growth. At the day 0 time point, the PAB cocktail inoculated sprouts were observed to have slightly brown tips; however, the sprouts had a firm appearance (Table 7). In contrast, the three samples of uninoculated sprouts displayed no initial signs of browning; however, two of the three uninoculated sprout samples were observed to have mold growth during harvest (Table 7). For the remaining 16 days, the PAB cocktail inoculated sprouts did not change from the day 0 time point with no further signs of browning, loss of firmness, or signs of mold growth (Table 7). Regarding the uninoculated sprouts, the sprouts did not experience a loss of firmness; however, one of three uninoculated sprout samples started to experience signs of browning within 4 days of harvest (Table 7). Furthermore, two of the three uninoculated sprout samples displayed signs of mold growth that appeared 4 and 13 days following harvest (Table 7).

Although the PAB cocktail inoculated alfalfa sprouts did not display drastic signs of spoilage, the slight browning effect that was observed may indicate that the PAB cocktail constituents have a minor antagonistic effect towards alfalfa sprouts; however, this minor antagonistic effect must be weighed against the fact that the PAB cocktail increased the final alfalfa sprout biomass by approximately 25% compared to uninoculated sprouts. Furthermore, the PAB cocktail inoculated sprouts were observed to not have any mold growth which may be attributed to previous findings that *Pantoea*, *Pseudomonas*, and *Priestia* have been found to have antagonistic effects towards plant pathogens and plant spoilage organisms such as molds (Chakraborty et al.; Dutkiewicz et al.: Part IV, 2016; Solanki et al., 2012; Quagliotto et al., 2009).

To further understand the potential antagonistic effects that each PAB cocktail constituent may have on alfalfa sprouts, genomic DNA was extracted from *Pantoea agglomerans*, *Pantoea sp. #3*, *Pseudomonas sp. #1*, and *Priestia megaterium #3*. The genomic DNA was then submitted for whole genome sequencing (WGS) to determine a more definitive identity of each PAB cocktail constituent and to elucidate genes associated with plant pathogenicity. Following WGS, each PAB cocktail constituent's genome was

assembled and annotated; however, a complete genome could not be assembled for any of the PAB constituents (Table 8).

Table 8: The BV-BRC genome ID associated with each PAB cocktail constituent in addition to the number of contigs that resulted from genome assembly, the estimated genome length, the GC content, and the number of open reading frames (ORFs).

Constituent	Genome ID	Number of Contigs	Genome Length (Mb)	GC Content (%)	Number of ORFs
JV6	53335.191	39	4.937	55.09	4,829
JV24	2800373.10	102	5.691	37.62	6,188
RG1	286.4911	63	5.979	60.10	5,550
RG18	53335.190	44	4.799	55.17	4,601

Based upon Table 8, the genomes of each PAB cocktail constituent were not completely assembled, which is indicated by the presence of 39, 102, 63, and 44 contigs for JV6, JV24, RG1, and RG18, respectively (Table 8). Contigs are contiguous segments of DNA sequence that represent fragments of an organism's genome; and under ideal circumstances, the results of WGS should yield one large contig representing the bacterial chromosome and multiple smaller contigs representing plasmids, if applicable. The inability to completely assemble these bacterial genomes may have resulted from repeating sequences found either in the bacterial chromosome or more commonly in plasmids (Robertson & Nash, 2018; Scott & Ely, 2015). Due to these repeating sequences, the read length generated using the short-read Illumina platform would not be long enough to accurately map the contigs together due to these repeating sequences. Therefore in future experimentation, long-read sequencing platforms developed by Oxford Nanopore or Pacific Biosciences (PacBio) should be used in tandem with the current short-read Illumina sequencing data to accurately assemble each PAB cocktail constituent's genome into a single or several contigs based upon the presence of plasmids (Scott & Ely, 2015).

Once each PAB cocktail constituent's genome was assembled and annotated, the identity of each constituent was determined by querying the BV-BRC database of reference genomes against the assembled PAB genomes to construct phylogenetic trees for JV6, RG1, and RG18 (Figure 11, Figure 12, Figure 13, and Figure 14).

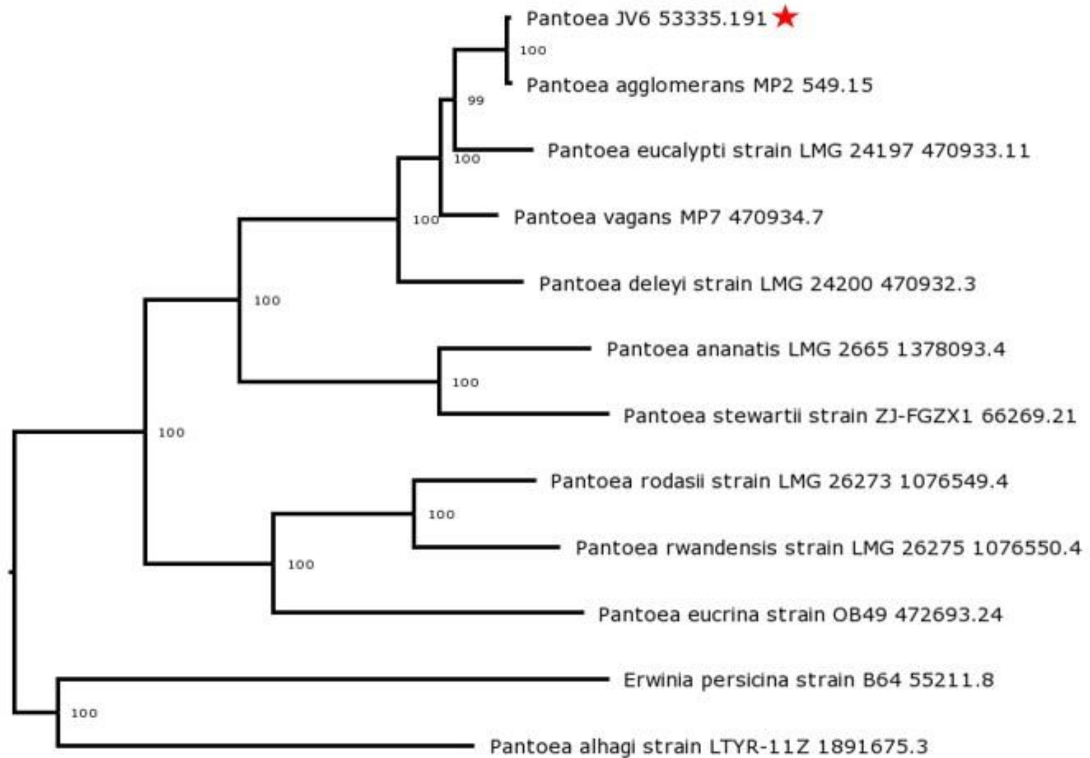

Figure 10: A phylogenetic tree comparing homology in the sequences of 100 single-copy genes of JV6 (Red Star) to genomes of *Pantoea spp.* available in the BV-BRC database.

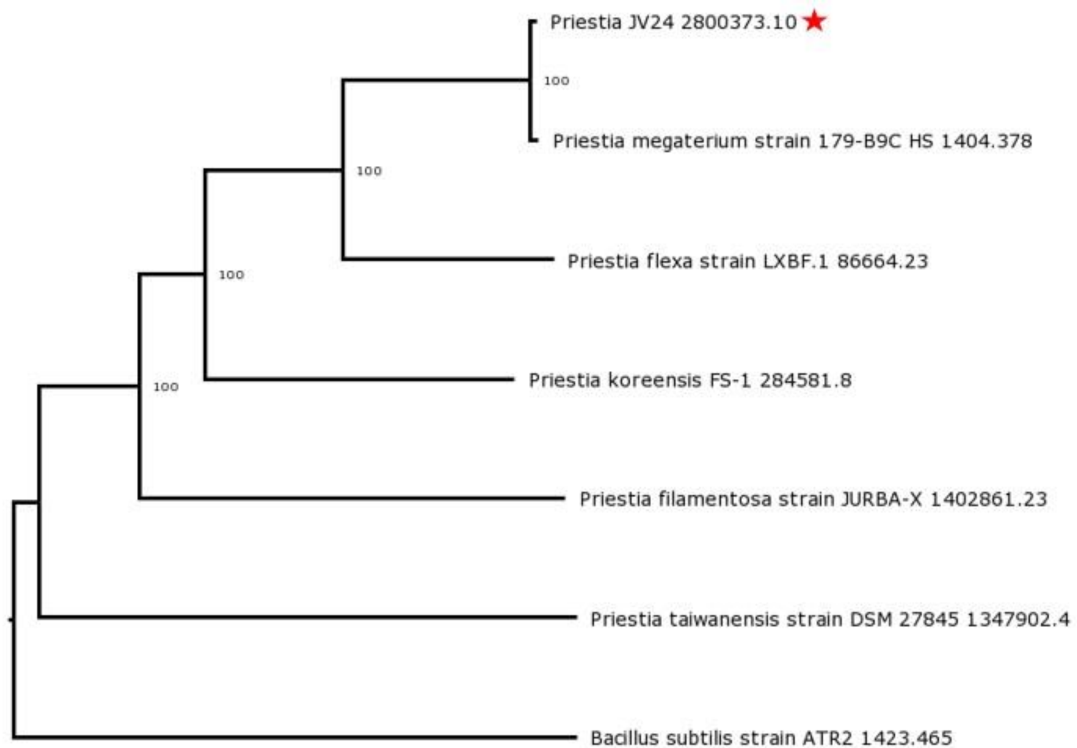

Figure 11: A phylogenetic tree comparing homology in the sequences of 100 single-copy genes of JV24 (Red Star) to genomes of *Priestia spp.* available in the BV-BRC database.

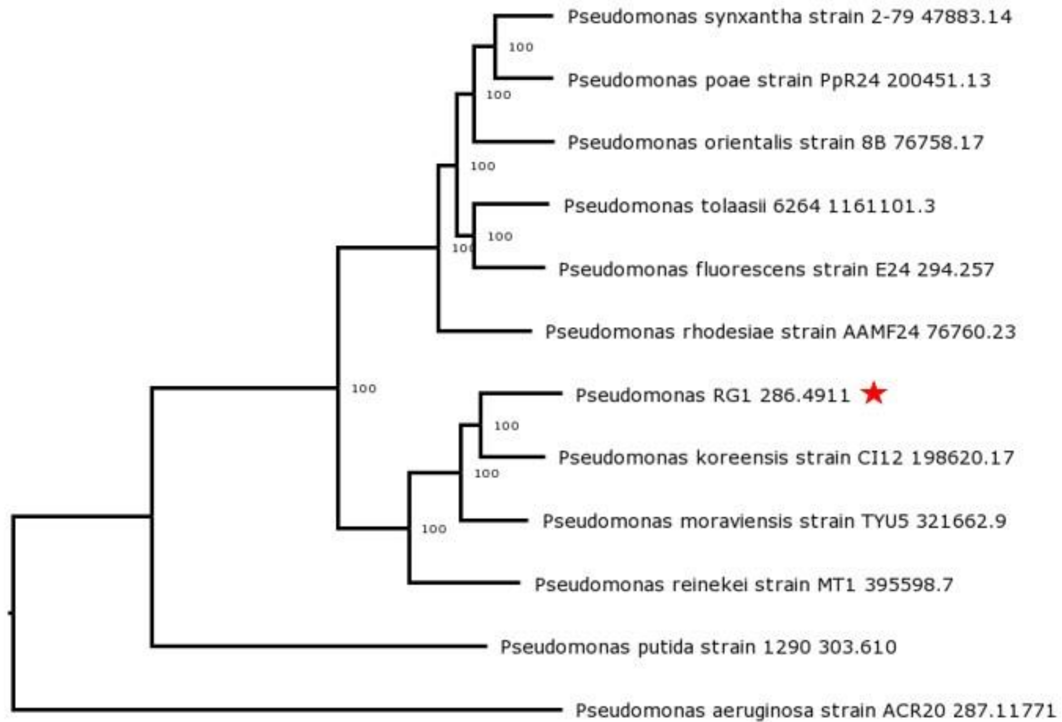

Figure 12: A phylogenetic tree comparing homology in the sequences of 100 single-copy genes of RG1 (Red Star) to genomes of *Pseudomonas spp.* available in the BV-BRC database.

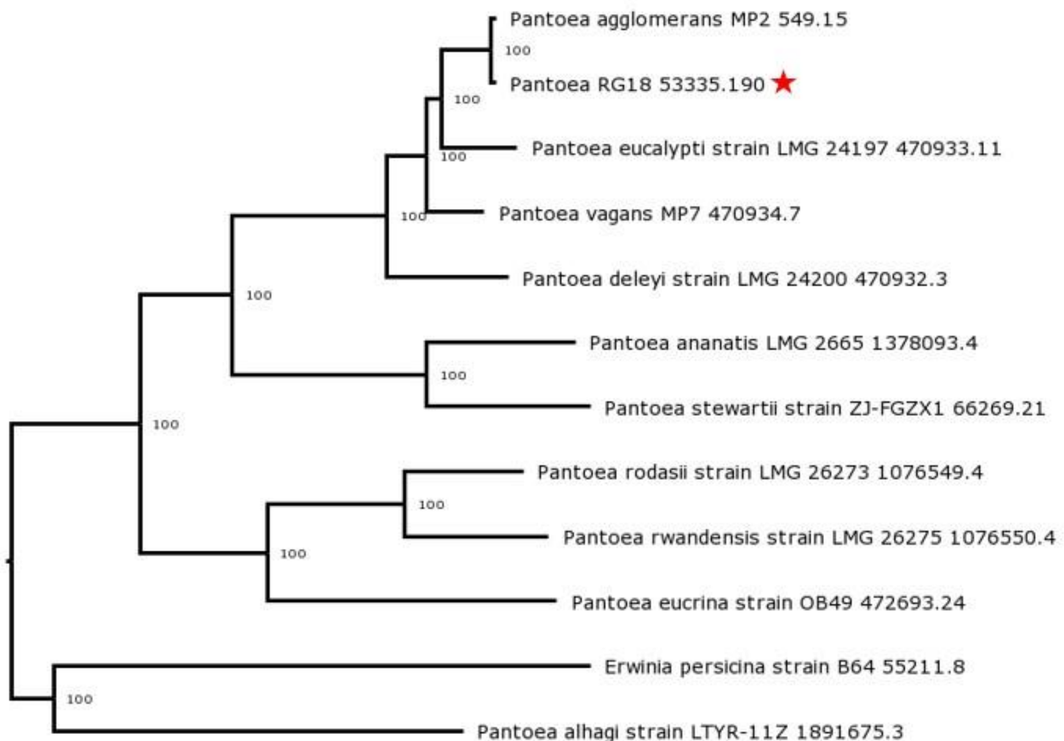

Figure 13: A phylogenetic tree comparing homology in the sequences of 100 single-copy genes of RG18 (Red Star) to genomes of *Pantoea spp.* available in the BV-BRC database.

Based on the constructed phylogenetic trees, JV6, JV24, RG1, and RG18 have been identified as *Pantoea agglomerans* JV6, *Priestia megaterium* JV24, *Pseudomonas koreensis* RG1, *Pantoea agglomerans* RG18 (Figure 11, Figure 12, Figure 13, and Figure 14). Although each phylogenetic tree indicates homology to a specific strain, the phylogenetic tree was not used to specify the exact strain of each PAB cocktail constituent; rather, the goal of the constructing the phylogenetic trees was to ascertain the identity of each PAB cocktail constituent to the species level. To identify the specific strain for each species, a more complete genome should be assembled in future experimentation following the aforementioned methods of long-read sequencing using either Oxford Nanopore or PacBio sequencing platforms.

Based upon the identity of the PAB cocktail constituents, *Priestia megaterium* JV24 and *Pseudomonas koreensis* were not screened further for plant pathogenicity characteristics. With regards to *P. megaterium*, there was no indication in peer-reviewed literature of plant pathogenicity. Rather, *P. megaterium* has been repeatedly used as a model organism for biotechnology applications and the species is a well-known plant-associated microorganism that displays plant-growth promoting properties (Bach et al., 2022; Biedendieck et al., 2021; Katsenios et al., 2021). Similar to *P. megaterium*, *P. koreensis* was not identified as a plant pathogen in peer-reviewed literature. Additionally, *P. koreensis* has been found to promote plant-growth through properties such as Nitrogen fixation and the production of phytohormones, and this species has also been identified as a biocontrol agent against fungal plant pathogens through the production of biosurfactants (Hultberg et al., 2010; Rafikova et al., 2016).

In contrast, *Pantoea agglomerans* is a known opportunistic pathogen of animals, plants, and humans. However, the species' pathogenicity varies based on the presence of plasmids containing pathogenicity islands that encode for the *hrp/hrc* type III secretion system (<u>H</u>ypersensitive <u>R</u>esponse <u>P</u>athogenicity/HR <u>C</u>onserved), and genes encoding virulence effector proteins (Alfano & Collmer, 2004; Dutkiewicz et al.: Part III, 2016). The *hrp* gene encodes the needle-like component of the type III secretion system that penetrates into host cells, injects effector proteins into the host cell, and the virulence effector proteins then negatively impact the host cell (Alfano & Collmer, 2004). In conjunction with Hrp, Hrc proteins are located at the base on the Hrp needle-like structure embedded in the bacterial membrane of the pathogenic cell, and the Hrc proteins function to allow the secretion of virulence effector proteins across the bacterial membrane to the Hrp needle-like structure and into the host cell (Alfano & Collmer, 2004; Tampakaki et al., 2010). With regards to virulence effector proteins, virulence effectors are quite diverse and classified under enzymes such as SUMO proteases, ubiquitin E3 ligases, phosphothreonine lyases, protein tyrosine phosphatases, papain-like cysteine proteases, staphopains, glycerophosphoryl phosphodiesterases, mono-ADP ribosyltransferases, and syringolide synthases (Tampakaki et al., 2010). Therefore, the genomes of *Pantoea agglomerans* JV6 and *Pantoea agglomerans* RG18 were screened for the *hrp/hrc* gene cluster in addition to

the aforementioned classes of effector proteins to assay for potential plant pathogenicity. Following analysis, the *hrp/hrc* gene cluster and effector proteins affiliated with type III secretion systems were not identified; however, a type VI secretion system was identified in both *P. agglomerans* JV6 and *P. agglomerans* RG18 (Table 9 and Table 10).

Table 9: The genes comprising a type VI secretion system and the copy number of each gene identified in *Pantoea agglomerans* JV6.

Gene and Gene Function	Copy Number
T6SS PAAR-repeat protein / RhaS protein	7
T6SS component Hcp	6
T6SS component TssM (IcmF/VasK)	3
T1SS secreted agglutinin RTX	2
T6SS AAA+ chaperone ClpV (TssH)	2
T6SS Serine/threonine protein kinase (PpkA)	2
T6SS associated component FIG00553474	2
T6SS associated component TagF (ImpM)	2
T6SS component TssA (ImpA)	2
T6SS component TssB (ImpB/VipA)	2

Table 10: The genes comprising a type VI secretion system and the copy number of each gene identified in *Pantoea agglomerans* RG18.

Gene and Gene Function	Copy Number
T6SS component Hcp	6
T6SS PAAR-repeat protein / RhaS protein	5
T6SS Serine/threonine protein kinase (PpkA)	2
T6SS associated component FIG00553474	2
T6SS component TagF (ImpM)	2
T6SS component TssM (IcmF/VasK)	2
T6SS forkhead associated domain protein (ImpI/VasC)	2
T6SS outer membrane component TssL (ImpK/VasF)	2

Although a type III secretion system was not identified, a type VI secretion system (T6SS) was identified in both *P. agglomerans* JV6 and *P. agglomerans* RG18; however, the presence of a T6SS does not necessarily guarantee plant pathogenicity. T6SSs are the most ubiquitous type of secretion system with estimates that 25% of all Gram-negative bacteria have a T6SS encoded in their genome (Kapitein & Mogk, 2013). T6SSs function as a contractible needle-like system that can puncture the membrane of a bacterial or eukaryotic cell and release effector proteins (Kapitein & Mogk, 2013). Once the effector protein is injected into the target cell, the effector will function as a cytotoxin that can be used either in bacterial competition or cause virulence in eukaryotes (Carobbi et al., 2021). One of the main components of the needle-like structure is the Hcp protein which comprises the barrel of the needle-like structure of T6SSs by forming linked hexameric rings (Kapitein & Mogk, 2013). Since this protein would be in high-abundance in this structure the copy number of the *hcp* gene would need to be relatively high which is verified by its high copy number of six in both *P. agglomerans* JV6 and *P. agglomerans* RG18 (Table 9 and Table 10). However, greater interest must be given towards the genes encoding PAAR-Rhs proteins that were also found in high numbers in both *P. agglomerans* JV6 and *P. agglomerans* RG18, 7 and 6 copies respectively. These PAAR-Rhs proteins serve as the aforementioned effector proteins that are injected into either a host eukaryotic cell or a competing bacterial cell. Once the PAAR-Rhs protein is injected into the target cell, the carboxy-terminus of the Rhs protein will have an antagonistic effect on the target cell due to a range of toxin domains that can occupy the carboxy-terminus such as DNase, RNase, deaminase, endonuclease, peptidase, pore-forming, or protein-modifying domains (Ma et al., 2017). However in the case of *Pantoea*, toxin domains with DNase, deaminase or protein-modifying functions have been identified in *P. ananatis* and *P. stewartii* while DNase activity was reported in *P. agglomerans* (Ma et al., 2017). Although *P. agglomerans* JV6 and RG18 do possess PAAR-Rhs effector genes, the functionality of these effectors is unknown and the functional purpose of T6SSs is quite vast including cell-to-cell communication, self from non-self identification, and interbacterial competition (Kapitein & Mogk, 2013).

Therefore, the evidence concerning *P. agglomerans* JV6 and *P. agglomerans* RG18 being plant pathogens is circumstantial following their identification and in-depth analysis of genes associated with plant-pathogenicity. Furthermore, the T6SS encoded in each organism's genome may be beneficial in interbacterial competition against *Salmonella* or other human pathogens *in planta*. Based upon the PAB cocktail's beneficial effect on alfalfa biomass development and the performance of the cocktail inoculated alfalfa in the shelf-life study, the PAB cocktail consisting of *P. agglomerans* JV6, *P. agglomerans* RG18, *P. koreensis* RG1, *P. megaterium* JV24, and *P. putida* KT-2440 was finalized and used in a coinfection experiment to assess the PAB cocktail's ability to mitigate *Salmonella* contamination in alfalfa sprouts.

2.3.5 *Salmonella* Typhimurium 4/74 Inoculation

Salmonella is known to colonize and grow quite prolifically on alfalfa sprouts on either the sprout surface or inside of the sprout tissue (Cui et al., 2018; Dong et al., 2003; Gandhi et al., 2001; Golberg et al., 2011). Therefore, surface decontaminated alfalfa seeds that were inoculated with *Salmonella* at an initial inoculum of ~4 log CFU/g displayed rapid growth within 24 hours to 6.5 log CFU/g and the *Salmonella* cell density stabilized at 8 log CFU/g after 9 days of incubation (Cui et al., 2018). Furthermore, Cui et al. reported that this exponential increase in *Salmonella* cell density occurred when the sprouts were grown on water-agar, a minimal medium, suggesting that the bacteria are being supported by exudates produced by the alfalfa. This result was verified by inoculating surface decontaminated and untreated FTL alfalfa seeds to ~ 4 log CFU/g of *Salmonella* Typhimurium 4/74 (Figure 5).

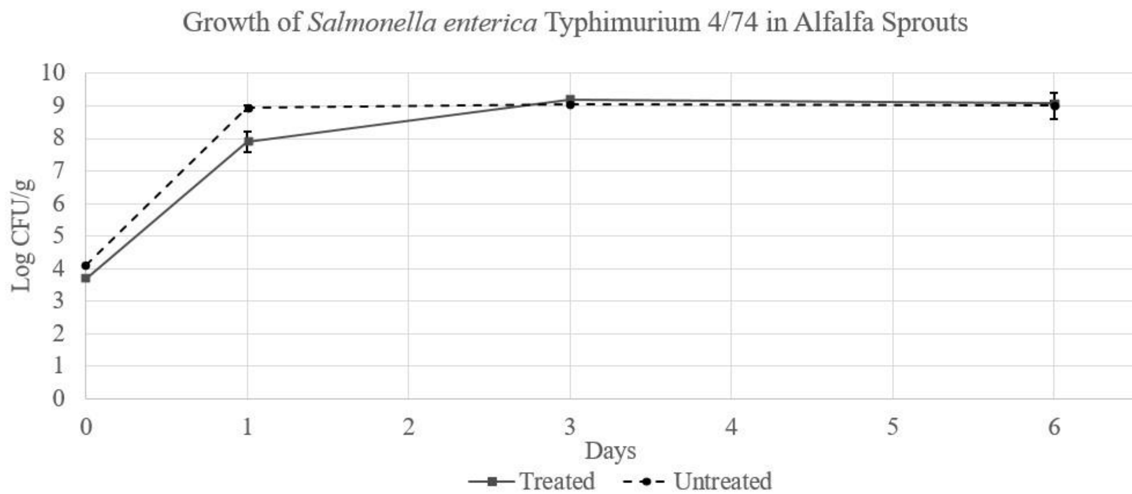

Figure 14: The cell density of *Salmonella* Typhimurium 4/74 extracted and enumerated from treated (surface decontaminated) and untreated Food to Live alfalfa seeds grown on water-agar over a six day period of time. n = 3 for both treated and untreated seeds. Error bars represent sample standard deviation.

Based on Figure 5, the cell density of *Salmonella* increased dramatically from the 4 log CFU/g inoculum to 7.9 log CFU/g and 8.9 log CFU/g for the treated and untreated seeds, respectively (Figure 5). Furthermore, the *Salmonella* cell density stabilized at ~9 log CFU/g for the treated and untreated seeds. In comparison to Cui et al., *Salmonella* is capable of a more rapid expansion in cell density ranging from a 4 to 5 log increase in cell density whereas Cui et al. only reported a 2.5 log increase within 24 hours (Cui et al., 2018). Additionally, the final cell density of *Salmonella* stabilized at 9 log CFU/g compared to 8 log CFU/g reported by Cui et al. (Cui et al., 2018). Therefore, the evidence further supports the notion that *Salmonella* not only survives on alfalfa seeds/sprouts, but *Salmonella* thrives exceptionally on alfalfa. In terms of the microbiota of the alfalfa being capable of controlling *Salmonella* contamination, the untreated FTL alfalfa seeds had a higher *Salmonella* density than the surface decontaminated seeds after 24 hours of incubation. One explanation for this result could be that the initial presence of PAB on

untreated FTL seeds was found to be ~3 log CFU/g and *Salmonella* was inoculated at 4 log CFU/g (Figure 4; Figure 5). Therefore, *Salmonella* would have had a 10-fold numerical advantage over the PAB which could have prevented the PAB from effectively competing with *Salmonella* for either space or limiting nutrients on the alfalfa.

2.3.6 *Salmonella*-Biocontrol Agent Coinfection

To test the *Salmonella* reduction efficacy of the biocontrol agent cocktail on alfalfa sprouts, the biocontrol agent cocktail was inoculated at a high cell density (7 log CFU/g) to provide the greatest competitive advantage in the favor of the plant-associated bacteria and positively stimulate alfalfa sprout biomass production. Therefore, 5 gram samples of FTL alfalfa sprouting seeds were inoculated with 7 log CFU/g of the PAB cocktail outlined in Table 4 and 3 log CFU/g *Salmonella* Typhimurium 4/74. Then, the *Salmonella* cell density was monitored with respect to time (Figure 15). This result was then compared to FTL alfalfa sprouting seeds that were inoculated with 3 log CFU/g of *Salmonella* Typhimurium 4/74 (Figure 15).

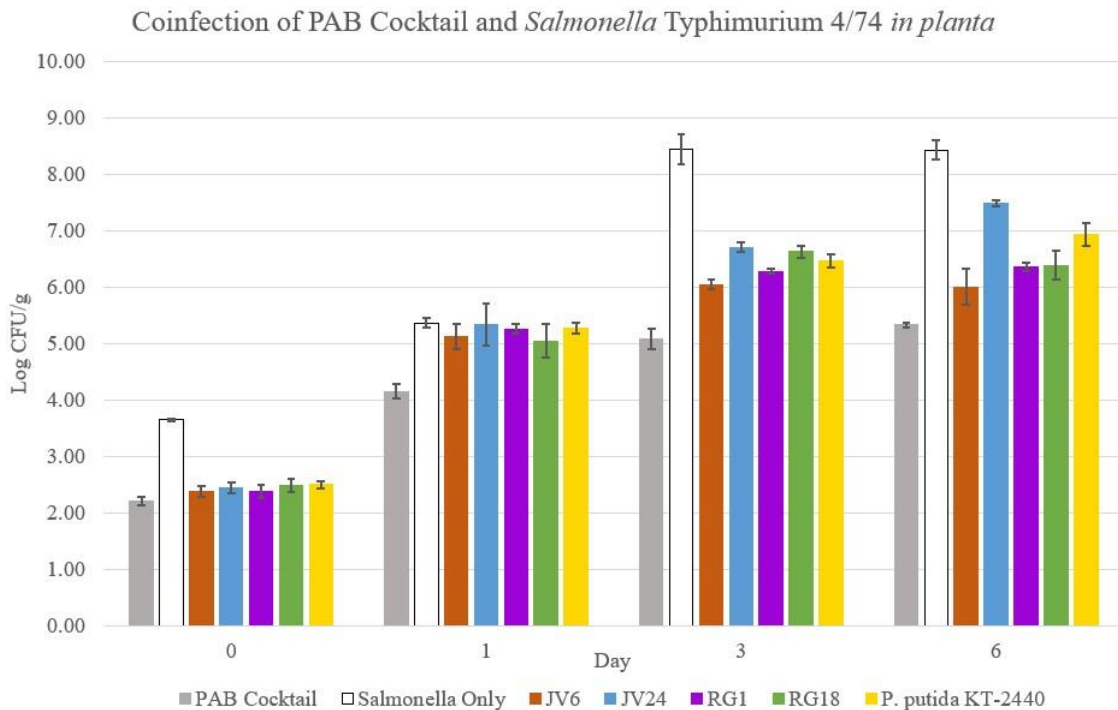

Figure 15: The cell density of *Salmonella* Typhimurium 4/74 extracted and enumerated from Food to Live alfalfa seeds that were inoculated with either the *Salmonella* & PAB cocktail, *Salmonella* only, or *Salmonella* & a PAB cocktail constituent. n = 3 for *Salmonella* inoculated seeds, n = 3 for *Salmonella*-PAB cocktail co-inoculated seeds, and n =3 for *Salmonella*-PAB cocktail constituent co-inoculated seeds. Error bars represent sample standard deviation.

Regarding the *Salmonella* inoculated alfalfa seeds, the *Salmonella* cell density increased from 3.64 log CFU/g to 5.36 log CFU/g within 24 hours, and this result differed

from a previous finding in which *Salmonella*'s cell density increased from 4.00 log CFU/g to 8 to 9 log CFU/g within 24 hours (Figure 14; Figure 15). Following three days of germination, the *Salmonella* cell density then increased to 8.44 log CFU/g and the cell density stabilized through the end of the six day germination period. Although the *Salmonella* cell density lagged somewhat within the first 24 hours compared to previous results, the final *Salmonella* cell density was similar to previous results and results obtained by Cui et al. (Figure 14) (Cui et al., 2018). In contrast, the alfalfa seeds co-inoculated with *Salmonella* and the PAB cocktail displayed a lower *Salmonella* cell density following 24 hours of germination (4.15 log CFU/g) (Figure 15). This cell density then increased to 5.08 log CFU/g and 5.33 log CFU/g after three and six days of germination, respectively. Therefore, the PAB cocktail was able to reduce the ability of *Salmonella* to colonize the alfalfa sprout surface throughout the entire sprout germination process, and the cocktail resulted in an overall 3.1 log reduction of *Salmonella* after six days of germination. To assay the effectiveness of each PAB cocktail constituent, each member of the PAB cocktail was co-inoculated individually with *Salmonella*.

According to Figure 15, the *Salmonella* cell density on seeds inoculated with *Pantoea agglomerans* JV6, *Priestia megaterium* JV24, *Pseudomonas koreensis* RG1, *Pantoea agglomerans* RG18, and *Pseudomonas putida* KT-2440 were less higher than the PAB cocktail with 5.05 to 5.33 log CFU/g following 24 hours of germination, 6.04 to 6.70 log CFU/g following 3 days of germination, and 6.0 to 7.49 log CFU/g following 6 days of germination (Figure 15). Therefore, *P. agglomerans* JV6, *P. megaterium* JV24, *P. koreensis* RG1, *P. agglomerans* RG18, and *P. putida* KT-2440 caused a 2.43, 0.94, 2.08, 2.04, and 1.50 log reduction, respectively, in the *Salmonella* cell density that was lower than the reduction caused by the PAB cocktail (Figure 15). Based on this observation, the total effectiveness of a diverse community of biocontrol agents was found to be more effective than a singular biocontrol agent. Although the PAB cocktail was found to be effective in reducing *Salmonella* cell density, *P. megaterium* JV24 and *P. putida* KT-2440 were found to not be as effective against controlling the cell density of *Salmonella* in comparison to the other PAB cocktail constituents. Therefore, the PAB cocktail that was formulated could be further developed to maximize the reduction of *Salmonella* while maintaining the cocktail's beneficial properties towards alfalfa biomass production.

Previous research regarding specific strains of biocontrol agents such as *Pseudomonas fluorescens* strain 2-79 and *Erwinia persicina* strain EUS78 found that these particular strains could cause a 5.5 log and 4.7 log reduction, respectively (Fett, 2005; Kim et al., 2020). Furthermore, the swarming bacterial species characterized as JV4 and JV10 may outcompete *Salmonella* for space on the alfalfa sprout surface due to these organism's coordinated swarming motility capability (Table 5). Therefore, these biocontrol agents could be substituted for other members of the biocontrol agent cocktail such as *P. megaterium* JV24 or simply be incorporated into the cocktail. Additionally, the current PAB cocktail constituents could be genetically modified to produce antimicrobial

compounds. *Pseudomonas putida* KT-2440 was specifically incorporated into the PAB cocktail because this strain of *P. putida* can be genetically modified to express foreign genes (Wu et al., 2011). A class of antimicrobial compounds named bacteriocins has drawn great attention because these compounds are relatively small antimicrobial peptides that are used by bacteria to kill other closely related bacteria (narrow-spectrum bacteriocins) or bacteria from other genera (broad-spectrum bacteriocins) (Negash & Tsehai, 2020). Schneider et al. have searched *Salmonella* genomic databases and identified five bacteriocins that target other *Salmonella* serotypes that they have been labeled as salmocins (Schneider et al., 2018). Of the five identified salmocins, SalE1a and SalE1b were found to display a high antimicrobial activity towards 99 *Salmonella* serotypes identified as major contributors towards human disease by the CDC (Schneider et al., 2018). Additionally, these salmocins were also expressed at high levels in the biopharmaceutical manufacturing host plant *Nicotiana benthamiana* (Schneider et al., 2018). Therefore, there is potential for salmocins to be used in other biotechnological applications such as transformation of the PAB cocktail constituent *P. putida* KT-2440 via a plasmid encoding the two salmocins. An alternative to adding bacterial species to the PAB cocktail or genetically modifying the cocktail's constituents would be the use of bacteriophages that would be co-inoculated with the PAB cocktail to further antagonize *Salmonella*. Ye et al. developed a biocontrol cocktail composed of seven bacteriophage and *Enterobacter asburiae* strain JX1 that was capable of reducing a cocktail of five *Salmonella* serotypes completely from mung bean sprouts after four days of germination (Ye et al., 2010). Therefore, the PAB cocktail could be co-inoculated with a bacteriophage cocktail that is specific towards *Salmonella*, which would be expected to have a more drastic reduction in *Salmonella* cell density compared to what is observed in Figure 15.

In summation, the biocontrol control developed using plant-associated bacteria was found to effectively and reduce the cell density of *Salmonella* on alfalfa sprouts while increasing alfalfa biomass production. However, there is great potential to further expand upon these results through refinements to the current biocontrol cocktail such as the addition or substitution of the PAB cocktail constituents with other known biocontrol agents, the genetic modification of PAB cocktail constituents to produce salmocins, or the use of a bacteriophage cocktail co-inoculated with the PAB cocktail.

2.4 Conclusions & Future Directions

The overall goal of this research was focussed upon isolating either a single plant-associated bacterial species or a community of plant-associated bacterial species that would be an effective biocontrol measure against *Salmonella* in alfalfa sprouts. To achieve this goal, a cocktail of plant-associated bacterial species isolated from either alfalfa seeds or sprouts was formulated based on each constituents' ability to enhance the production of alfalfa biomass. The resulting cocktail was composed of *Pantoea agglomerans* JV6, *Priestia megaterium* JV24, *Pseudomonas koreensis* RG1, *Pantoea agglomerans* RG18, and *Pseudomonas putida* KT-2440. With a complex community of plant-associated

bacteria, the PAB cocktail was found to increase alfalfa biomass production by 25% when compared to uninoculated seeds while not causing a detriment to the overall shelf-life of harvested alfalfa sprouts. In terms of the PAB cocktail's ability to control *Salmonella*, the PAB cocktail was able to reduce the ability of *Salmonella* to colonize the alfalfa sprout surface throughout the entire sprout germination process resulting in an overall 3.1 log reduction of *Salmonella* after six days of germination. However, the developed biocontrol cocktail was found to not completely eliminate *Salmonella* from alfalfa sprouts, thus sprouts with a reduced amount of *Salmonella* would still be in violation of the Produce Rule (Produce Rule, 2015). As a result, the application of a biocontrol PAB cocktail should not be used as the only control measure to limit *Salmonella* contamination; rather, biocontrol should be implemented to work in synchrony with seed decontamination and post-harvest mitigation strategies in a hurdle approach to sprout safety. Additionally, the PAB cocktail could undergo further development by exploring opportunities such as supplementing the PAB cocktail with known biocontrol agents, genetically modifying the current PAB cocktail constituents to express antimicrobial compounds with specificity towards *Salmonella*, and/or use a bacteriophage cocktail in tandem with the PAB cocktail to effectively eliminate *Salmonella* from the sprout surface.

Chapter 3: Characterization of Essential Genes to the Infection and Colonization of Alfalfa by *Salmonella* Typhimurium 4/74 Through Transposon Insertion Sequencing

3.1 Introduction

3.1.1 Current Knowledge on *Salmonella* Genes Essential for Alfalfa Infection and Colonization

To understand the close association that *Salmonella* has with alfalfa sprouts, previous work has focussed primarily on transcriptomic or proteomic approaches to determine essential genes that are required for *Salmonella* to colonize/grow on alfalfa sprouts. Regarding transcriptomics, several studies have shown that genes responsible for actions such as motility, biofilm formation, stress response, virulence, amino acid biosynthesis, and antimicrobial resistance were elevated compared to the reference transcriptome (Brankatschk et al., 2014; Zheng et al., 2021). In terms of proteomic studies, Wang et al. has shown that proteins specifically responsible for amino acid biosynthesis were found in higher abundance compared to the reference proteome which further supports the same observation reported by Brankatschk et al.'s RNA-seq study (Brankatschk et al., 2014; Wang et al., 2015). Furthermore, classical genetics studies have been performed in which genes responsible for key metabolic functions of *Salmonella* were knocked out and the mutants were assayed in alfalfa sprouts. Based on these studies, genes responsible for functions such as *de novo* amino acid biosynthesis, siderophore biosynthesis, attachment to plant cell walls, O-antigen biosynthesis, and the isomerization of fructose-6-phosphate to mannose-6-phosphate were identified as essential towards *Salmonella*'s colonization and proliferation on alfalfa sprouts (Hao et al., 2012; Kwan et al., 2015; Kwan et al., 2018; Tan et al., 2016).

3.1.2 Using Functional Genomics to Drive Biocontrol Agent Development

Using these results, the development of biocontrol agents can be further refined by screening biocontrol agents to target essential metabolites produced by either *Salmonella* or alfalfa that are critical for the infection of *Salmonella*. Kwan et al. have shown that the isomerization of fructose-6-phosphate to mannose-6-phosphate is critical for *Salmonella* to grow in alfalfa (Kwan et al., 2018). Therefore, a biocontrol agent can be screened for an enhanced metabolic consumption of fructose or mannose that would limit these compounds in alfalfa. Additionally, *de novo* amino acid biosynthesis was found to also play a significant role in the growth of *Salmonella* on alfalfa (Kwan et al., 2015). As a result, nitrogen sources such as urea, uric acid, homoserine, and aspartate that have been identified in the exudate of alfalfa seeds would be potential metabolic targets for a biocontrol agent (Lukman et al., 2011). Therefore, TnSeq, which has not been performed in alfalfa, could be used to further verify the aforementioned results and/or build upon our understanding of the ability of *Salmonella* to infect and colonize alfalfa sprouts.

3.1.3 The Basis of Transposon Insertion Sequencing, Potential Deficiencies, and its Application in Studying *Salmonella* Gene Essentiality *in vitro*, *in vivo*, and *in planta*

Transposon Insertion Sequencing (TnSeq) is a methodology of functional genomics that uses transposon insertion mutagenesis and subsequent sequencing to understand the impact of changes in a bacteria's genome on the fitness of that bacteria under experimental conditions (Lariviere & Batut, 2022). Transposons are highly regulated, short DNA segments that can excise themselves and translocate randomly within a genome (Lariviere & Batut, 2022). The random insertion of transposons would be used to generate mutants of *Salmonella* where the transposon has either inserted itself directly into a gene rendering it ineffective or the transposon has inserted itself into regulatory regions that will negatively impact a gene's expression (Davie et al., 2015; Lariviere & Batut, 2022). To do so, the transposon is inserted into *Salmonella* through the following methods: a plasmid encoding the transposon enters the host organism via electroporation or conjugation, or the transposon in association with a transposome enters the host organism via electroporation (Lee et al., 2013; Licandro-Seraut et al., 2012; Varushkina et al., 2021). Once a transposon mutant library is created of *Salmonella*, the mutant library can then be subjected to a particular growth condition (Lariviere & Batut, 2022). Since each individual mutant will have at least one gene disrupted by a transposon, the diversity of the mutant library will change with respect to time i.e. less competitive mutants will be lower in abundance and more competitive mutants will have a higher abundance (Lariviere & Batut, 2022). To analyze the change in abundance of each mutant, polymerase chain reaction (PCR) and next-generation sequencing (NGS) would be used to amplify and subsequently sequence the transposon-genome junction (Lariviere & Batut, 2022). Based on the sequencing results, the relative abundance of each mutant can be cross-referenced to the initial abundance of mutants at the zero time point or the reference mutant library (Lariviere & Batut, 2022). Therefore, conditionally essential genes for growth under specific experimental conditions can be correlated to mutants whose relative abundance decreased with respect to time because the loss of gene functionality conferred a negative fitness on that particular mutant.

One limitation of TnSeq is that only non-essential genes are studied simply because mutants with insertions in essential genes would not have survived the library preparation process (Cain et al., 2020). Additionally, transposon libraries must have an adequate amount of saturation i.e. the percentage of the genome that has a transposon insertion. DeJesus et al. notes that a saturation has to be high enough where each non-essential gene has at least several insertions; therefore, a saturation of 27 to 42% of a host's genome can be sufficient to perform TnSeq experiments (DeJesus et al., 2017). However, a high saturation of the host genome cannot guarantee that all non-essential genes will be completely knocked-out by transposon mutagenesis because *Salmonella* is known to possess genes that complement each other and the virulence plasmids of *Salmonella* have been found to have a heterogeneous copy number (Porwollik et al., 2014: Sánchez-Romero

et al., 2020). As a result, a TnSeq study may not accurately measure the effect of all non-essential genes in *Salmonella*.

Despite these limitations, TnSeq has been utilized to elucidate *Salmonella* gene essentiality under a range of experimental conditions such as *in vitro* studies, in various animal models, in food, and in plants.

In vitro studies of transposon mutant libraries of *Salmonella* have been directed towards the pathogen's ability to survive the harsh intracellular environment of macrophages or mononuclear phagocytic cells. Specifically, Fitzsimmons et al. and Karash et al. have identified specific genes associated with the inactivation of Nitric Oxide and Hydrogen Peroxide degradation, respectively, as potential survival strategies of *Salmonella* following phagocytosis by innate immune cells (Fitzsimmons et al., 2018; Karash et al., 2017). Furthermore, Mandal & Kwon identified two subunits of ATP synthase (alpha and delta subunits) and a magnesium/nickel/cobalt transport protein as essential genes responsible for the survival of *Salmonella* under desiccating conditions such as in low-moisture foods (Mandal & Kwon, 2017). In an applied TnSeq study, genes associated with DNA recombination and repair, biosynthesis of lipopolysaccharide, and osmoregulation were found to impact the survival of *Salmonella* on low-moisture pistachios (Jayeola et al., 2020). Therefore, there is potential to translate TnSeq experiments to more complex systems such as food; furthermore, TnSeq has been utilized to study *Salmonella* in animal and plant models.

Chaudhuri et al. developed a library of 7,702 Tn5 transposon mutants in *Salmonella* Typhimurium, inoculated three categories of food-producing animals: chickens, pigs, and calves, and analyzed *Salmonella* genes essential to oral infection (Chaudhuri et al., 2013). Based on their analysis, genes associated with the type III secretion system, fimbriae production, and stress response such as heat shock and oxidative stress were found to be essential in the oral infection of food-producing animals by *Salmonella* (Chaudhuri et al., 2013). In relation to TnSeq performed *in planta*, TnSeq's application concerning *Salmonella* has been quite limited; however, several studies have been conducted in tomato plants. For *Salmonella* to persist on tomato plants, carbohydrates were identified as the main carbon source; however, the exact carbohydrates required were unknown (de Moraes et al., 2017). The main factor identified for *Salmonella*'s growth on tomato plants were genes associated with *de novo* amino acid biosynthesis to yield amino acids such as arginine, glutamine, glutamate, methionine, tryptophan, threonine, and branched chain amino acids (de Moraes et al., 2017). However, this requirement of *de novo* amino acid biosynthesis was conditional based on the presence of other microorganisms in the tomato plant. When green tomatoes were coinfected with *Salmonella* and *Pectobacterium carotovorum*, a phytopathogen that causes soft rot disease, the soft rot condition created by *P. carotovorum* led to the presence of amino acids in the environment that by-passed the requirement for *Salmonella* to carry out *de novo* amino acid biosynthesis to persist on tomatoes (George et al., 2018). Therefore, TnSeq studies may provide insight into genes

that are essential towards *Salmonella*'s growth under specific environmental conditions. However, when performing TnSeq studies in plants or animals, the complexity of these systems can be impacted by changes such as the presence of the organism's natural microbiota or pathogenic species that could either negatively or positively impact the fitness of *Salmonella*. As a result, the data obtained from TnSeq studies should not be considered the final determination of conditional gene essentiality.

Ultimately, the goal of TnSeq studies is to identify potential *Salmonella* genes that play a role in *Salmonella*'s survival under specific experimental conditions; however, these broad-spectrum studies must be followed by classical genetics studies that target the identified genes to determine essentiality (Sawitzke et al., 2013). Therefore, a gene knockout must be performed in *Salmonella* where genetic engineering is utilized to cause an identified essential gene to lose all functionality, and a pure culture of the mutant is generated (Sawitzke et al., 2013). To test the hypothesis of a gene's conditionally essential function, the *Salmonella* mutant and wild-type *Salmonella* are subjected to the experimental condition, and the fitness of the mutant is compared to wild-type *Salmonella*. If the *Salmonella* mutant displays reduced fitness compared to the wild-type strain, then the gene that was knocked out can be considered essential for *Salmonella* to grow under those specific growth conditions. Furthermore, a rescue experiment can be performed where the essential gene located on a plasmid is restored in the mutant, and the rescued mutant and wild-type *Salmonella* are then resubjected to the experimental condition (Barker et al., 2014). If the fitness of the rescued mutant is comparable to wild-type *Salmonella*, the gene is confirmed further to be essential towards the growth of *Salmonella* in that condition.

3.1.4 Objectives

The overall goal of performing this TnSeq study was to identify metabolites produced by alfalfa or metabolites inherent to the sprout germination environment that can be targeted for consumption by biocontrol agents to limit the growth of *Salmonella*. To achieve this goal, the following six objectives were established:

1. Create a transposon mutant library in *Salmonella* Typhimurium 4/74 that had a sufficient number of transposon mutants to thoroughly saturate the *Salmonella* genome with mutations.
2. Inoculate alfalfa seeds with the transposon mutant library, and harvest the bacteria from the plant surface at the 0, 1, 3, and 6 day time points during sprout germination to capture the change in mutant diversity.
3. Extract the genomic DNA of the *Salmonella* that was harvested during each time point, perform a TnSeq library preparation protocol, and submit the prepared library for Illumina sequencing.
4. From the sequencing results, determine the relative abundance of each transposon mutant for each time point. Determine the log change in relative abundance of each

mutant at the 1, 3, and 6 day time points when compared to the initial abundance at day 0.

5. Identify mutants that experienced a negative log change in relative abundance, and determine the function of the gene containing the transposon insertion.
6. Identified genes responsible for metabolism will be selected for further analysis utilizing classical genetics studies.

3.2 Materials & Methods

3.2.1 Development of Transposon Mutant Library

To develop electrocompetent *Salmonella* cells, five 250 mL sterile flasks containing 50 mL of Super Optimal Broth (SOB) were inoculated with two drops of an overnight culture of *Salmonella* Typhimurium 4/74. The inoculated broth was then incubated at 37°C and shaken at 250 RPM until an OD_{600} (Optical Density at a wavelength of 600 nm) between 0.5 and 0.7 was achieved. Following incubation, each culture was transferred to a chilled 50 mL Falcon tube and the cultures were incubated on ice for 15 minutes. The cells were then centrifuged for 10 minutes at 5000 RPM and 4°C. Following centrifugation, the resulting supernatant was discarded. 10 mL of 100% glycerol was then added to each Falcon tube, the *Salmonella* cells were resuspended, and the cells were aggregated into a single 50 mL Falcon tube. The cells were then centrifuged again for 10 minutes at 5000 RPM and 4°C, the supernatant was discarded, the cells were resuspended in 50 mL of 100% glycerol, and the cells were centrifuged a third time following the same parameters. The supernatant was discarded from the Falcon tube and the electrocompetent *Salmonella* cells were then resuspended in the residual 100% glycerol. The resuspended cells were then stored as 1 mL aliquots at -80°C.

To prepare the transposon mutant library, a 1 mL aliquot of electrocompetent *Salmonella* Typhimurium 4/74 cells was thawed on ice for 10 minutes. Additionally, an electroporation cuvette and a 1.5 mL microcentrifuge tube were also chilled on ice. 25 µL of the thawed electrocompetent cells were then added to the chilled microcentrifuge tube in addition to 0.5 µL of the EZ-Tn5 <Kan-2> Tnp Transposome (Lucigen, Middleton, WI), and the solution was mixed by pipetting up and down. The Transposome-cell mixture was then transferred to the chilled electroporation cuvette, the cuvette was transferred to the MicroPulser Electroporator (BioRad, Hercules, CA), and the *Salmonella* cells were electroporated using the bacterial setting Ec1 of the MicroPulser. Following electroporation, 975 µL of Super Optimal Broth with catabolite repression (SOC) medium was added to the electroporated cells, the cells were transferred to a 15 mL Falcon tube, the *Salmonella* cells were recovered by incubation at 37°C with 250 RPM of shaking for 1 hour. After the cells had recovered, 100 µL of the undiluted SOC culture was plated on an LB-Kanamycin (LB-Kan) plate and the LB-Kan plate was incubated at 37°C for 24 hours.

Following incubation of the LB-Kan plate, the CFU/mL of transposon mutants was determined and the transformation efficiency was calculated based on the CFU of transposon mutants and the amount of transposome used in pmol. To divide the transposon

mutants into distinct sub-libraries, the SOC culture was diluted threefold and the entire culture was plated on LB-Kan plates in 100 µL. The LB-Kan plates were then incubated at 37°C for 24 hours and the number of mutants per LB-Kan plate was determined. Based on the number of mutants per plate, the mutants were allocated to one of seven distinct sub-libraries that would contain roughly 3,000 mutants per sublibrary. To achieve the required number of mutants to complete seven sub-libraries, the electroporation protocol was performed three times. Once each LB-Kan plate had been assigned to a specific sublibrary, the *Salmonella* cells were harvested by adding 5 mL of LB to each plate and a sterile spreader was used to scrape the cells from the agar surface. The cells were then aseptically transferred to a 50 mL Falcon tube and centrifuged for 10 minutes at 5000 RPM. The supernatant was discarded and the cells were resuspended in 30 mL of a 1:1 ratio of LB and 50% glycerol. The resuspended cells of each sublibrary were then divided into 1 mL aliquots and each aliquot was stored at -80°C until inoculation into alfalfa.

3.2.2 Inoculation of Alfalfa with Transposon Mutant Sublibraries

To inoculate alfalfa seeds with each respective transposon mutant sublibrary, 15 gram aliquots of Food to Live alfalfa seeds were surface decontaminated and each aliquot was inoculated with a single transposon mutant sublibrary to 4 log CFU/g. The inoculated seeds were then grown under large-scale growth conditions for six days. At the day 0, 1, 3, and 6 time points, the alfalfa seeds and/or sprouts were harvested, transferred into sterile flasks, and *Salmonella* cells were extracted from the plant surface. To isolate the bacterial cells, approximately 25 to 400 mL of PHG solution, completely immerse the plant tissue, and 100 5 mm glass beads were added to each flask, and the flasks were shaken at 200 RPM for 30 minutes (Kingsley & Bohlool, 1981). With volumes in excess of 50 mL of PHG solution, the resulting *Salmonella* extract was transferred to a sterile 400 mL Bio-Bottle (ThermoFisher, Waltham, MA) and centrifuged at 5,000 RPM for 15 minutes. The supernatant was discarded into biological waste; 25 mL of PBS was added to the Bio-Bottle and vortexed; and the concentrated bacterial sample was transferred to a sterile 50 mL Falcon tube. For volumes less than or equal to 50 mL of PHG solution, the resulting *Salmonella* extract was transferred directly to a sterile 50 mL Falcon tube. From the *Salmonella* extract, a serial dilution was performed to yield a 10^{-1}, 10^{-3}, 10^{-5}, and 10^{-7} dilution. LB-Kan agar plates were spread-plated using the prepared dilutions, and the plates were incubated at 37°C for 24 hours.

After incubation, the CFUs were counted on the LB-Kan plates, the CFU/mL of the *Salmonella* extract was determined, and the CFU/g of the PAB from the isolated alfalfa was determined using the measured volume of *Salmonella* extract and mass of alfalfa harvested.

Once the CFU/g was determined, the *Salmonella* extract for the day 0, 1, 3, and 6 time points and respective sublibrary was prepared for long-term frozen storage. The *Salmonella* extract was centrifuged at 5000 RPM for 15 minutes to concentrate the *Salmonella* cells, the resulting supernatant was discarded to biological waste, and the cells

were resuspended in 4 mL of a 1:1 ratio of LB broth and 50% glycerol solution. The resuspended cells were then stored as 1 mL aliquots at -80°C until library preparation.

3.2.3 Aggregation of Sublibraries and Preparation of Cells for gDNA Extraction

The *Salmonella* cells for each time point and respective transposon mutant sublibrary were thawed on ice for 15 minutes. For the day 0, 1, 3, and 6 time points, the *Salmonella* cell concentrations were normalized based on the CFU/mL of each 1 mL aliquot of resuspended cells. Since the day 0 time point's cell concentration was low (10^4 CFU), the cells were enumerated by transferring 4 mL of the normalized *Salmonella* cells to two large glass dishes, 4 mL per dish, containing approximately 600 mL of LB-Kan agar. The cells were then distributed over the surface of the agar with a sterile spreader and the glass dishes were incubated at 37°C for 24 hours. Following incubation, 30 mL of LB was added to the surface of each dish, the cells were scraped off the agar surface with a sterile spreader, and the cells were aseptically transferred to two separate 50 mL Falcon tubes.The cells were then centrifuged at 5000 RPM for 15 minutes, the supernatant was decanted, and the cells in both tubes were aggregated in 15 mL of LB. To remove plant debris from the day 1, 3, and 6 time points' normalized cells, the cells were passed through a 5 µm mixed cellulose esters (MCE) membrane filter (Millipore-Sigma, Darmstadt, Germany) via vacuum filtration. The resulting filtrate was then centrifuged at 5000 RPM for 15 minutes, the supernatant was decanted, and the *Salmonella* cells were resuspended in 4 mL of LB.

The gDNA of the normalized *Salmonella* cells at each time point was then extracted using the NucleoSpin Tissue kit (TakaraBio, Kusatsu, Shiga, Japan) following the manufacturer's instructions. After extraction, the concentration of dsDNA was determined using a Qubit fluorometer (ThermoFisher, Waltham, MA). Following quantification, the gDNA was stored at -20°C until further use. Two different transposon library preparation methodologies were tested; therefore, two separate gDNA extractions were performed for the day 0, 1, 3, and 6 time points.

3.2.4 Preparation of Samples for TnSeq Analysis: Methodology #1

The first transposon library preparation methodology was adapted from a protocol published by Karash et al. to create a Tn-Seq amplicon library, and the general workflow of the adapted protocol is outlined in Figure 16 (Karash et al., 2019).

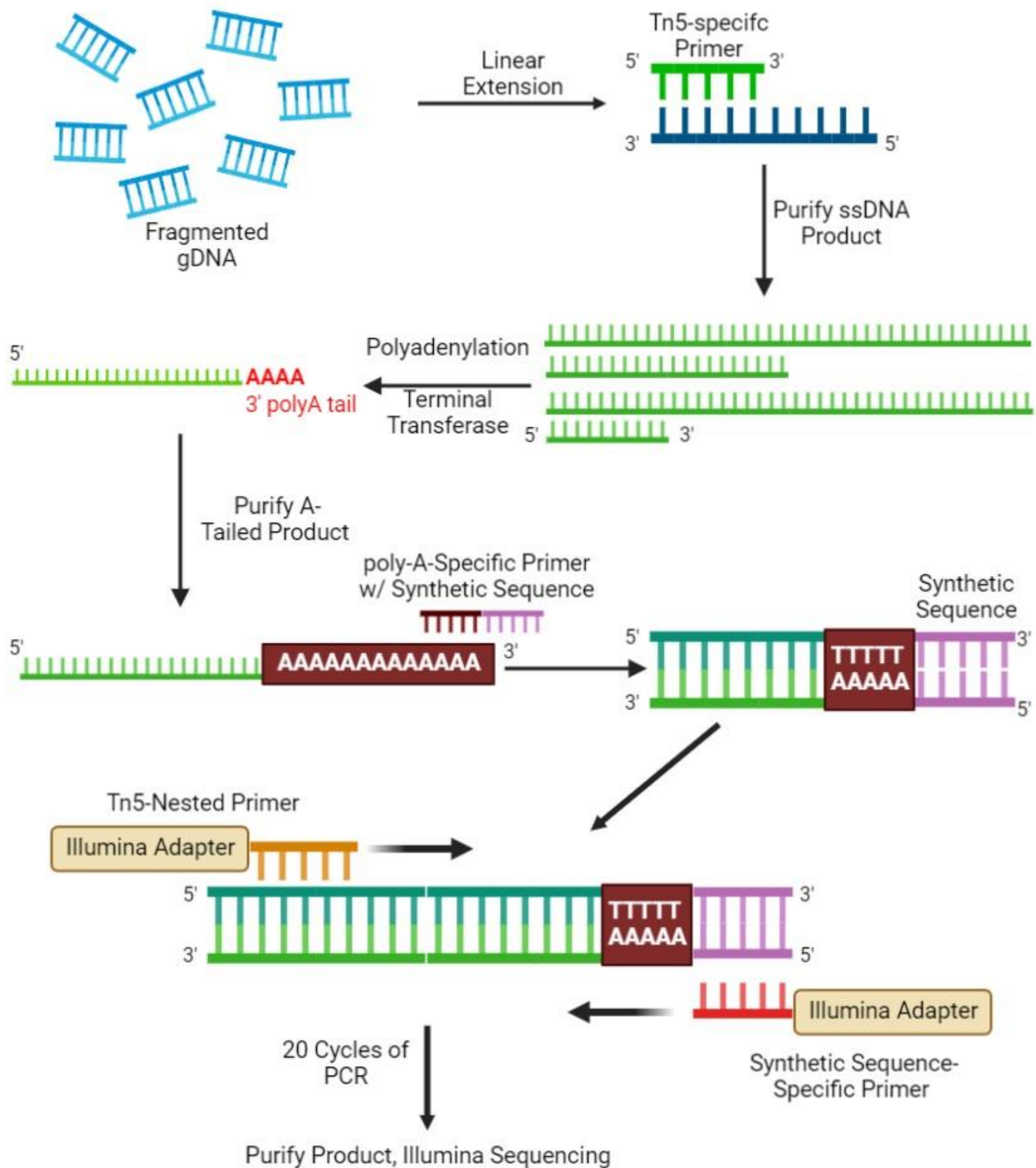

Figure 16: A general workflow of the protocol adapted from Karash et al. used to create a Tn-Seq amplicon library for Illumina sequencing. Image created using BioRender.com.

To mitigate potential problems associated with secondary structure of the extracted gDNA, 1.5 µg of gDNA (15 - 30 ng/µL) for each time point was submitted to the University

of Minnesota Genomics Center (UMGC, Minneapolis, MN), and the gDNA was sheared to an average size of 300 base pairs (bp) via Covaris Shearing (Covaris, Woburn, MA). Upon reception of the sheared gDNA, the gDNA was stored at -20°C to prevent DNA degradation.

The primers used for the remainder of the protocol are outlined in Table 11. Primer 1 was obtained from the EZ-Tn5 <Kan-2> Tnp Transposome kit (Lucigen, Middleton, WI), and the primer was used to create transposon-specific linear extension products from the 5' end of the Tn5 transposon (Figure 16, Table 11). Primer 2 was used to create a dsDNA product from the poly-A tailed linear extension product that contained a synthetic DNA sequence that is not present in the *Salmonella* genome to facilitate the amplification of the dsDNA product (Figure 16, Table 11). Primer 3 and Primer 4 were then used to amplify the Tn-Seq amplicon with a partial Illumina handle required for the Illumina sequencing service offered through Genewiz (Azenta Life Sciences, Plainfield, NJ) where Primer 3 was a nested primer specific to the Tn5 transposon sequence and Primer 4 was specific to the synthetic sequence (Figure 16, Table 11).

Table 11: Primers used for linear extension, dsDNA synthesis, and amplification of transposon specific sequences to prepare a Tn-Seq amplicon library.

Primer Name	Primer Sequence (5' → 3')	Reference
Primer 1	GCAATGTAACATCAGAGATTTTGAG	Lucigen, Middleton, WI
Primer 2	TATGAGGACGAATCTCCCGCTTATATTTTT TTTTTTTTTTTTTTTTTTTTTTTTT	Xu et al., 2009
Primer 3	ACACTCTTTCCCTACACGACGCTCTTCCGA TCTNNNNNNNNNNNNCGATGATGGTTGA GATGTGTATAAGAGACAG	Azenta Life Sciences, Plainfield, NJ
Primer 4	GACTGGAGTTCAGACGTGTGCTCTTCCGA TCTNNNNNNNNNNNNTATGAGGACGAAT CTCCCGCTTATA	Azenta Life Sciences, Plainfield, NJ Xu et al., 2009

Using Primer 1, a linear extension reaction was prepared according to Table 12, and fifty cycles of a linear extension were performed following the thermocycler conditions outlined in Table 13 (Table 11, Table 12. A linear extension negative control reaction was also prepared using day 6 sheared gDNA without the Taq 2x Master Mix added to the reaction. This control would be used through the remainder of the protocol to ensure that primers were effectively removed during reaction clean-ups and to check that preparation of the final Tn-Seq amplicon was dependent on the linear extension reaction i.e. background gDNA would not be amplified with the Tn-Seq amplicons. One limitation of

this negative control was that the control used sheared gDNA for the Day 6 time point which contained the Tn5 sequence. A more optimal negative control would have been the use of sheared gDNA extracted from wild-type *Salmonella* Typhimurium 4/74 which would not have had the Tn5 sequence in its genome.

Table 12: Linear extension reaction preparation components and the conditions for each linear extension reaction to create linear extension products from the 5’ end of the Tn5 transposon.

Reaction Preparation		Linear Extension Conditions		
Reagent	Volume (µL)	Temperature	Number of PCR Cycles	Time
Sheared gDNA	2.0	95°C	1	2 min
Primer 1 (20 µM)	1.0	95°C	50	30 sec
Taq 2x Master Mix	25	62°C		45 sec
Nuclease-Free H_2O	22	72°C		10 sec
Total	50	4°C	1	Hold

Following linear extension, the resulting product was purified using the DNA Clean and Concentrator-5 kit (Zymo Research, Irvine, CA), and eluted into 12 µL of 65°C elution buffer. After extraction, the concentration of dsDNA and single-stranded (ss) DNA was determined using a Qubit fluorometer (ThermoFisher, Waltham, MA).

Using the linear extension product, an A-tailing reaction was prepared as outlined in Table 13. A 30:1 mole ratio of deoxyadenosine triphosphate (dATP) to dideoxycytidine triphosphate (ddCTP) was used to create an average A-tail length of thirty deoxyadenosine bases. On average, the terminal transferase would add thirty deoxyadenosine bases to the 3’ end of the linear extension product before adding a dideoxycytidine base which cannot undergo further extension because the dideoxycytidine lacks a 3’ hydroxyl group.

Table 13: A-tailing reaction components used to A-tail linear extension products.

Reagent	Volume (µL)
Linear Extension Product	5.0
10x TdT Buffer	2.0
2.5 mM $CoCl_2$	2.0
10 mM dATP	3.0

1 mM ddCTP	1.0
Terminal Transferase	0.5
Nuclease-Free H_2O	6.5
Total	20

The A-tailing reaction was then incubated for 1 hour at 37°C to allow for A-tailing to occur and the reaction was halted by denaturing terminal transferase at 75°C for 10 minutes. The resulting product was then purified using the DNA Clean and Concentrator-5 kit (Zymo Research, Irvine, CA), and eluted into 20 µL of 65°C elution buffer. After extraction, the concentration of dsDNA and ssDNA was determined using a Qubit fluorometer (ThermoFisher, Waltham, MA).

Following A-tailing, the complementary strand to the ssDNA product was synthesized and the Tn-Seq amplicon was amplified via a two-step reaction process. Using Primer 2 outlined in Table 11, a dsDNA synthesis reaction was prepared and the reaction was performed using the thermocycler conditions outlined in Table 14.

Table 14: dsDNA synthesis reaction preparation components and the reaction conditions programmed to the thermocycler.

Reaction Preparation		Reaction Conditions		
Reagent	Volume (µL)	Temperature	Number of PCR Cycles	Time
A-Tailed ssDNA	2.0	95°C	1	2 min
Primer 2 (10 µM)	1.0	95°C		30 sec
Taq 2x Master Mix	25	43°C	10	45 sec
Nuclease-Free H_2O	20	72°C		10 sec
Total	48	72°C	1	1 min
		4°C	1	Hold

Following dsDNA synthesis, the samples were placed immediately on ice, then 2 µL of a solution composed equally of Primer 3 (10 µM) and Primer 4 (10 µM) was added to each reaction. The samples were then returned to the thermocycler and the Tn-Seq amplicon was amplified according to the PCR conditions detailed in Table 15.

Table 15: Thermocycler conditions used to amplify the Tn-Seq amplicon.

Temperature	Number of Cycles	Time
95°C	1	2 min
95°C		30 sec
54°C	20	45 sec
72°C		10 sec
4°C	1	Hold

The resulting amplicon was then purified and size-selected (200 to 500 bp) using NEBNext sample purification magnetic beads (New England Biolabs, Ipswich, MA). After vortexing the NEBNext sample purification beads, 10 µL of sample purification beads were added to the 50 µL PCR reaction. The sample was then incubated at room temperature for 5 minutes. After incubation, the sample tube was placed in a magnetic rack and the tube was left undisturbed for 5 minutes. After 5 minutes, the liquid in the tube was transferred to a clean 200 µL PCR tube, and an additional 10 µL of NEBNext sample purification beads were added to the tube. The tube was then placed back into the magnetic rack and left undisturbed for 5 minutes. Following 5 minutes, the supernatant was discarded into a clean 200 µL PCR tube, and the NEBNext sample purification bead pellet was washed twice with 200 µL of 80% ethanol. The purification beads were then air-dried for 5 minutes to remove any residual ethanol. The purification beads were then resuspended in 22 µL of 65°C elution buffer, and the tube was incubated at room temperature for 2 minutes. The tube was then placed back into the magnetic rack, left undisturbed for 2 minutes, and 20 µL of purified, size-selected Tn-Seq amplicon was transferred to a clean 200 µL PCR tube.

Following size-selection and purification, the concentration of dsDNA was determined using a Qubit fluorometer (ThermoFisher, Waltham, MA). Then, 1 µL of each product was separated by size via gel electrophoresis, and a 100 bp ladder was used for the DNA size standard. The gel was composed of 1.5% agarose gel containing 10 µL of SYBR-Safe gel stain (ThermoFisher, Waltham, MA) in Tris-Borate-EDTA (TBE) buffer (90 mM Tris Base, 90 mM Boric Acid, 2 mM EDTA). The gel was submerged in TBE buffer and electrophoresis was performed for 1 hour and 45 minutes using 120 V. After electrophoresis, the gel was visualized under UV-light using a ChemiDoc XRS visualization system (BioRad, Hercules, CA).

Following quantification and visualization of the Tn-Seq amplicon for the 0, 1, 3 and 6 day time points, each sample's concentration was normalized to 20 ng/µL. Then 25 µL of each sample was submitted to Genewiz (Azenta Life Sciences, Plainfield, NJ) using their Amplicon-EZ Illumina sequencing service to generate at least 50,000 sequencing

reads as an initial quality control check of the Tn-Seq amplicon library before further Illumina sequencing was performed.

3.2.5 Preparation of Samples for TnSeq Analysis: Methodology #2

The second transposon library preparation methodology was performed via Fasteris SA, a third-party laboratory service (Genesupport SA, Plan-les-Ouates, Geneva, Switzerland). Per the Fasteris SA requirements, unsheared gDNA for the day 0, 1, 3, and 6 time points was normalized to a concentration of 40 to 60 ng/µL. Then 50 µL of each sample (2 to 3 µg of gDNA) was submitted to Fasteris SA for the third-party laboratory service to perform a TnSeq library preparation and subsequent Illumina sequencing.

3.2.6 Data Analysis of TnSeq Results

Upon completion of Amplicon-EZ Illumina sequencing, each sequencing file was downloaded and then uploaded to the Department of Energy Systems Biology Knowledgebase (Kbase) (US Department of Energy, Washington DC). In addition to each sequencing file, a reference genome of *Salmonella* Typhimurium 4/74 submitted to GenBank by Richardson et al. was acquired from Kegg Genome (Kyoto University, Kyoto, Japan) and uploaded to Kbase (Richardson et al., 2011). In Kbase, each sequencing file was then aligned to the reference genome using Bowtie2 v2.3.2 using the following parameters: phred33 for alignment quality scoring, local fast alignment, trim 45 bases from 5' end, trim 175 bases from 3' end, minimum fragment length of 10 bases, and maximum fragment length of 40 bases. Once alignment was completed in Kbase, a Qualimap report was generated to provide insight into the quality of the resulting TnSeq alignment, and each alignment was checked for sufficient coverage of the *Salmonella* genome before further analysis was performed.

3.3 Results & Discussion

3.3.1 Development of Transposon Mutant Library

For a transposon mutant library to be created, the target organism i.e. *Salmonella* must have either a transposon-transposase complex (transposome) or a plasmid that contains the transposon sequence and encodes the transposase enzyme inserted inside of the target cell through methodologies such as conjugation or transformation (Hamer et al., 2001; Simon et al., 1983). The transposon mutant library created for this study utilized the EZ-Tn5 <Kan-2> kit purchased from Lucigen (Middleton, WI) which was composed of the Tn5 transposon that was associated with a transposase. Electrocompetent *Salmonella* cells were then electroporated and transformed with the EZ-Tn5 <Kan-2> transposome to yield *Salmonella* Tn5 mutants. This methodology was performed three times to construct a library of 21,428 *Salmonella* Tn5 mutants that were divided amongst seven sublibraries of approximately 3,000 mutants (Table 16).

Table 16: The recorded time constant and resulting transformation efficiency for each transformation experiment performed. The number of *Salmonella* Tn5 mutants allocated to each sublibrary.

Transformation	Time Constant (ms)	Transformation Efficiency (CFU/pmol)	Sublibrary	Number of Mutants
1	4.60	1.828×10^5	1	3168
2	4.90	9.4×10^4	2	3218
3	4.40	5.44×10^4	3	3014
			4	3119
			5	2944
			6	2964
			7	3001

Following three electroporation experiments, a Tn5 library of 21,428 mutants was constructed to ensure that each gene in the *Salmonella* Typhimurium 4/74 genome would hypothetically have four Tn5 insertions on average (Richardson et al., 2011). However, the true distribution of insertions in the mutant library would vary such as a higher number of Tn5 mutants in large open reading frames (ORFs), a lower number of Tn5 mutants in smaller ORFs, and zero representation of essential genes (Jacobs et al., 2003).

Furthermore, the transformation efficiency of the performed transformations ranged from 1.828×10^5 CFU/pmol to 5.44×10^4 CFU/pmol while Lucigen estimated a transformation efficiency of $>10^5$ CFU/pmol (Lucigen, Middleton, WI) indicating that the transformation protocol could be optimized to increase the transformation efficiency of *Salmonella*. According to O'Callaghan & Charbit, the transformation efficiency of *Salmonella* could be increased 10 to 100 fold through the introduction of mutations to *galE* or the *hsd* restriction modification, respectively (O'Callaghan & Charbit, 1990). However, the introduction of mutations to the *Salmonella* genome to increase the efficiency of generating transposon mutants may not be viable since restriction modification systems play a critical role in the self-defense of bacterial species (Vasu & Nagaraja, 2013). Therefore, the elimination of these systems would have a detrimental effect on the mutants' overall fitness. Alternatively, a different methodology could have been utilized to prepare electrocompetent *Salmonella* cells using a pulsing buffer (PB) composed of Tris, Sucrose and $MgCl_2$ (Eynard, 2000). According to Eynard, the magnesium assists to enhance the viability of fragile cells and the sucrose increases the external cellular osmolarity during membrane permeabilization that further protects the cell (Eynard, 2000). Therefore, these

changes to the electrocompetent cell preparation methodology may lead to an increase in transformation efficiency to streamline the process of mutant library preparation.

3.3.2 Inoculation of Alfalfa with Transposon Mutant Sublibraries

Salmonella is known to colonize and grow quite prolifically on alfalfa sprouts on either the sprout surface or inside of the sprout tissue (Cui et al., 2018; Dong et al., 2003; Gandhi et al., 2001; Golberg et al., 2011). According to Cui et al., surface decontaminated alfalfa seeds that were inoculated with *Salmonella* at an initial inoculum of ~4 log CFU/g displayed rapid growth within 24 hours to 6.5 log CFU/g and the *Salmonella* cell density stabilized at 8 log CFU/g after 9 days of incubation (Cui et al., 2018).

Prior to performing the inoculation, the number of mutants used per inoculation had to be determined to effectively represent each mutant in the Tn5 library. Mandal & Kwon and Fitzsimmons et al. utilized Tn5 libraries of *Salmonella* Typhimurium that were composed of ~370,000 and ~230,000 Tn5 mutants, respectively, for *in vitro* experiments (Fitzsimmons et al., 2018; Mandal & Kwon, 2017). However, the use of these super-saturated mutant libraries would not be ideal when using an inoculum of ~4 log CFU/g because approximately 1 CFU/mutant would be applied to the alfalfa seeds which could lead to poor representation of the mutant library (Calculation 1). Furthermore, the inoculation of the alfalfa seeds with the entire mutant library of 21,428 mutants would also present problems with sufficient representation of each mutant if minor variation occurred during aggregation of the library.

$$2 \times 10^4 \, CFU/g \times 15\, g = 300{,}000\, CFU$$

$$\frac{300{,}000\, CFU}{300{,}000\, mutants} = 1\, CFU/mutant$$

$$\frac{300{,}000\, CFU}{21{,}000\, mutants} = \sim 14\, CFU/mutant$$

$$\frac{300{,}000\, CFU}{3{,}000\, mutants} = 100\, CFU/mutant$$

Calculation 1: Example calculation used to justify the use of 3,000 mutant sublibraries for inoculation of alfalfa sprouts.

Based on Calculation 1, the use of 3,000 mutant sublibraries was required to effectively represent each mutant to approximately 100 CFU/mutant at the initial day 0 time point (Calculation 1). Therefore, each Tn5 mutant sublibrary was inoculated on 15 grams of surface decontaminated FTL alfalfa seeds at an initial inoculum of 4 to 5 log CFU/g and the inoculated seeds were germinated for six days (Figure 17).

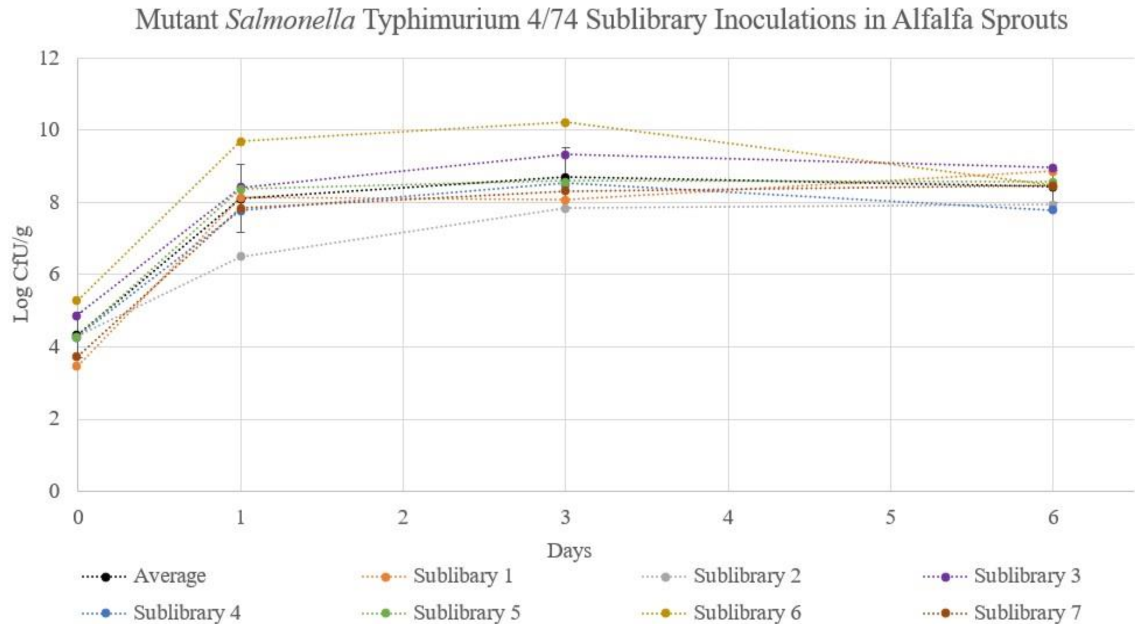

Figure 17: The cell density of seven sublibraries of Tn5 mutant *Salmonella* Typhimurium 4/74 cells extracted and enumerated from surface decontaminated Food to Live alfalfa seeds grown on water-agar over a six day period of time. n = 1 for all samples. Error bars represent standard deviation for sample averages.

On average, the cell density of the Tn5 mutant *Salmonella* increased dramatically from the 4.3 log CFU/g inoculum to 8.1 log CFU/g within 24 hours (Figure 17). Furthermore, the average Tn5 mutant *Salmonella* cell density increased to ~8.7 log CFU/g after three days of germination and the average cell density then decreased to ~8.4 log CFU/g after six days of germination (Figure 17). However, of the seven sublibraries inoculated into the alfalfa sprouts, Sublibrary 2 was observed to have ~1.5 log CFU/g less cell density within 24 hours while Sublibrary 6 was observed to achieve a cell density nearly 2 log CFU/g higher as compared to the average cell density (Figure 17). However, the cell density for both sublibrary samples stabilized at 8.0 to 8.5 log CFU/g following six days of germination, which was in-line the average cell density (Figure 17). In comparison to Cui et al., the Tn5 mutant *Salmonella* sublibraries were capable of a more rapid expansion in average cell density ranging from a 4 to 5 log increase in cell density whereas Cui et al. only reported a 2.5 log increase within 24 hours (Cui et al., 2018). Additionally, the final cell density of *Salmonella* stabilized at 8 to 9 log CFU/g, and this result was comparable to that reported by Cui et al. for wild-type *Salmonella* (Cui et al., 2018). Therefore, the evidence supports the notion that *Salmonella* not only survives on alfalfa seeds/sprouts, but *Salmonella* thrives exceptionally on alfalfa. Furthermore, the inoculation experiments resulted in sufficient quantities of Tn5 mutant *Salmonella* cells to perform gDNA extractions and perform the necessary TnSeq library preparation methodologies to visualize the changes in the Tn5 mutant library as alfalfa seed germination progressed.

3.3.3 Data Analysis of TnSeq Study

As outlined in Figure 16, the methodology adapted from Karash et al. comprised a three step TnSeq library preparation protocol that used a linear extension step to increase the amount of sequences that contained the Tn5 insertion site; an A-tailing procedure was then conducted to add a poly-A tail of approximately 30 adenosine bases to the 3’ end of the linear extension product; then two sequential PCR reactions were performed to synthesize the complementary strand of the linear extension product and amplify the resulting double-stranded product (Figure 16). Throughout this workflow, the concentration of both ssDNA and dsDNA was measured to determine the efficacy of each step in the workflow compared to a negative control consisting of day 6 sheared gDNA without the Taq 2x Master Mix added to the reaction during the initial linear extension step in the workflow (Table 17).

Table 17: The concentration of ssDNA and dsDNA that was measured throughout the TnSeq library preparation workflow. The negative control consists of day 6 sheared gDNA without the Taq 2x Master Mix added during the initial linear extension step.

Linear Extension			A-Tailing Reaction		
Sample Name	[dsDNA] ng/µL	[ssDNA] ng/µL	Sample Name	[dsDNA] ng/µL	[ssDNA] ng/µL
Day 0	32.8	123	Day 0	9.04	25.0
Day 1	19.6	72.4	Day 1	4.42	12.3
Day 3	21.8	77	Day 3	4.78	13.5
Day 6	47.6	156	Day 6	4.64	16.2
(-) Control	0.132	0.380	(-) Control	Too Low	0.300

Complementary Strand Synthesis and Amplification	
Sample Name	[dsDNA] ng/µL
Day 0	27.8
Day 1	28.9
Day 3	21.4
Day 6	20.1
(-) Control	1.12

According to Table 17, the linear extension was effective in creating ssDNA products that were observed in at least 100 times the abundance compared to the negative control indicating that the recorded ssDNA concentrations for the day 0, 1, 3, and 6 samples were not attributed to primer contamination (Table 17). However, one limitation of this negative control was that the control used sheared gDNA for the Day 6 time point which contained the Tn5 sequence. A more optimal negative control would have been the use of sheared gDNA extracted from wild-type *Salmonella* Typhimurium 4/74 which would not have had the Tn5 sequence in its genome. Therefore, the observed ssDNA linear extension product may have resulted from potential off-target extension of non-Tn5 sequences in the *Salmonella* genome. Additionally, the concentration of dsDNA for the linear extension product ranged from 19.6 to 47.6 ng/µL for the samples; however, the negative control had a reading of 0.132 ng/µL (Table 17). These readings likely resulted from the single-stranded linear extension product base pairing to form small secondary structures such as hairpins that would bind the dsDNA Qubit dye and then produce a signal in the Qubit.

Following the A-tailing reaction and subsequent clean-up, the concentration of ssDNA product was dramatically lower with a 33.8%, 28.3%, 29.2%, and 17.3% recovery of ssDNA for the day 0, 1, 3, and 6 samples, respectively (Table 17). Due to this low recovery, a potential bottleneck may have been introduced to each sample where ssDNA sequences that were present in a relatively low abundance may have been lost during the clean-up stage resulting in sequences present in a relatively high abundance to be over represented in the sample.

Once the A-tailed products were purified, the complementary strand of the ssDNA product was synthesized and the dsDNA product was amplified. A size selection step was then performed to clean-up the PCR reaction and select for TnSeq amplicons that were 200 to 500 bp in size. To check the effectiveness of the size-selected product, 1 µL of each amplicon was distributed by size on a gel via electrophoresis (Figure 18).

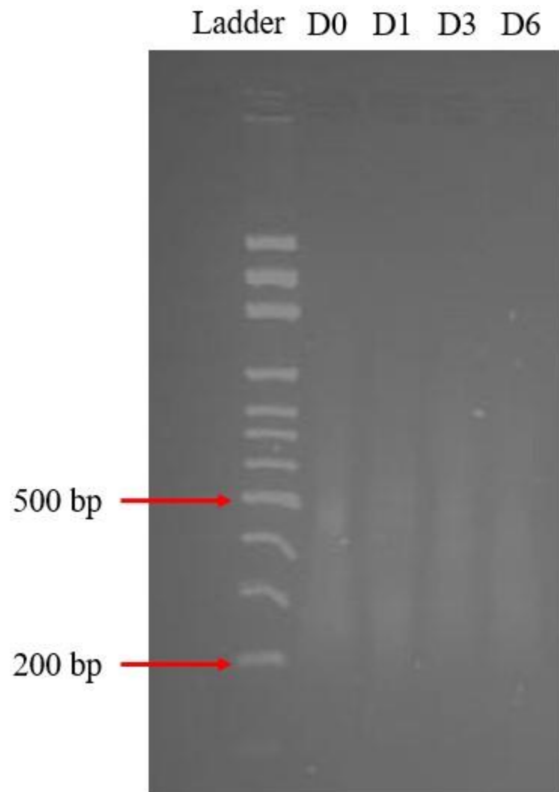

Figure 18: A TBE gel image displaying TnSeq amplicons of the 0, 1, 3, and 6 day samples. For size comparison, a standardized 100 base pair (bp) DNA ladder was used with the desired size range (200 bp to 500 bp) indicated by red arrows.

Based on Figure 18, the resulting TnSeq amplicons for the day 0, 1, 3, and 6 samples had a wider size distribution than expected with ranges from 200 bp to 900 bp (Figure 18). Therefore, the magnetic bead size selection methodology used was not effective in achieving the desired amplicon size distribution. In future experimentation, the final size selection should be adjusted to adhere to the recommendations by Karash et al. which was the use of gel purification (Karash et al., 2019). By using gel purification, the entire TnSeq amplicon sample would be visualized on the gel, the desired size range excised from the gel, and a gel purification kit could be used to obtain a pure sample of the TnSeq amplicon in the desired size range. Additionally, the concentration of the TnSeq amplicon was rather low indicated by the low intensity of each samples' bands compared to the 100 bp DNA ladder (Figure 18). The final Qubit measurements for each TnSeq amplicon sample were 27.8 ng/µL, 28.9 ng/µL, 21.4 ng/µL, and 20.1 ng/µL for the day 0, 1, 3, and 6 samples, respectively (Table 17). However, the quantity and concentration of each sample's amplicon was sufficient to submit the amplicons for Illumina sequencing via Genewiz (Azenta Life Sciences, Plainfield, NJ).

Upon reception of the Illumina sequencing results for the TnSeq samples, the day 0 sample was analyzed for sufficient saturation of the *Salmonella* genome with unique transposon insertion sites and the overall quality of the sequencing results was determined

(Figure 19). DeJesus et al. notes that a saturation has to be sufficient where each non-essential gene has at least several insertions; therefore, a saturation of 27 to 42% of a host's genome can be sufficient to perform TnSeq experiments (DeJesus et al., 2017).

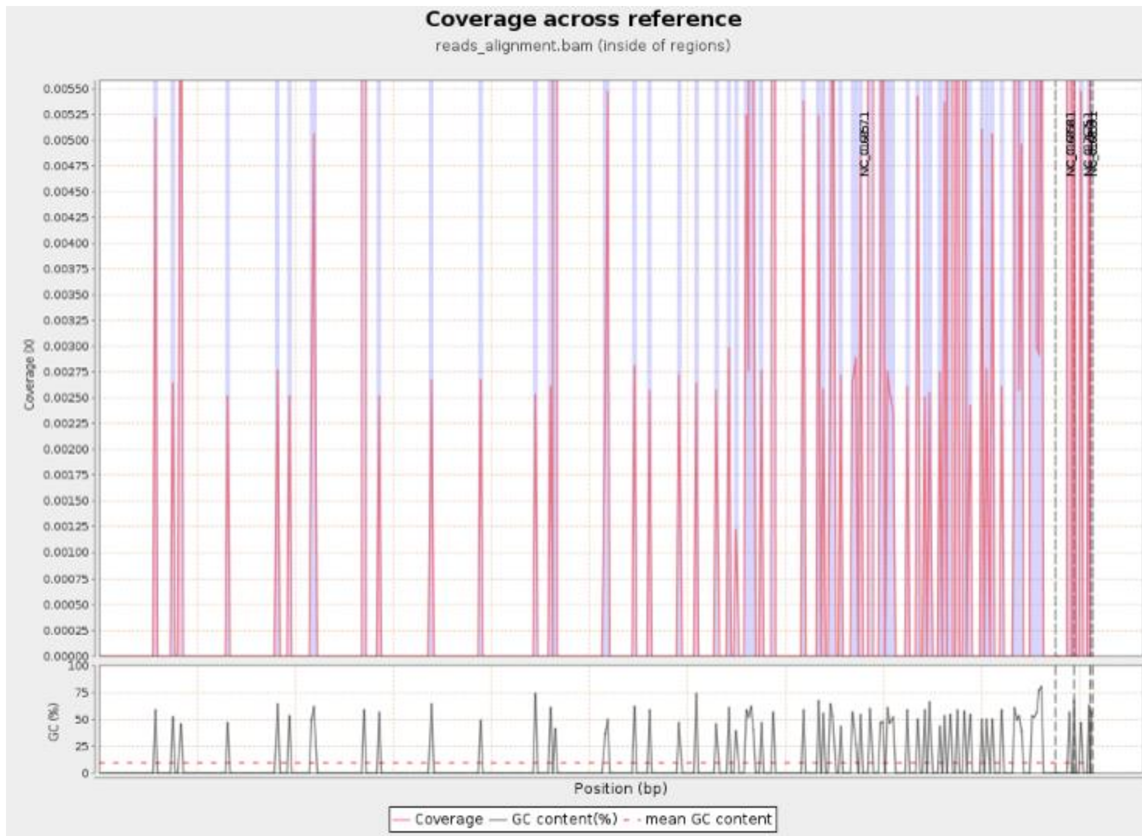

Figure 19: The coverage of the day 0 amplicon sequences that align with a reference *Salmonella* Typhimurium 4/74 genome, and the GC content of each aligned sequence. Each peak represents a Tn5 insertion site in the reference genome.

According to Figure 19, each peak corresponds to a transposon insertion site in the *Salmonella* Typhimurium 4/74 genome; however, only ~3,100 unique insertions of the 21,428 mutant library were recorded by Qualimap (Figure 19). Furthermore, the distribution of insertion sites was not uniform across the reference genome; rather, identified insertion sites were found to form hotspots around particular loci in the genome leading to a low coverage of the reference genome (Figure 19). As a result, the day 0 sample was deemed to not be usable as a reference to compare against the day 1, 3, and 6 samples because the day 0 sample only reflected one seventh of the mutants assayed in the TnSeq study and the hot spotting effects observed indicated potential bias. The cause of this bias remains unknown; however, PCR bias was hypothesized to be the most likely contributor. PCR bias results from the unequal amplification of a template sequence due to intrinsic factors such as differences in the amplification efficiency of different template sequences or self-annealing of the most abundant template in later stages of amplification that inhibits

amplification (Acinas et al., 2005). The methodology utilized to prepare the TnSeq amplicon libraries relied heavily on PCR-based methods to increase the amount of Tn5-specific sequences. As a result, the linear extension step in the methodology required 50 cycles of extension to create ssDNA sequences corresponding to Tn5 insertion sites. However, the efficiency of extension may have been favored towards the ~3,100 Tn5 insertion sites observed in the day 0 sample data. As a result, the ~3,100 Tn5 insertion sequences would have constituted a higher proportion of the sample following linear extension. Then the sequences in a higher abundance would be overrepresented in the sample following each clean-up used in the workflow as was observed with the clean-up after the A-tailing reactions (Table 19).

Since the methodology adapted from Karash et al. was unsuccessful, a second transposon library preparation methodology was performed. However, due to time constraints, Fasteris SA, a third-party laboratory service (Genesupport SA, Plan-les-Ouates, Geneva, Switzerland) was contracted to perform a library preparation and subsequent Illumina sequencing of the day 0, 1, 3, and 6 TnSeq samples. The processing of these samples is on-going and the results will likely not be available prior to the submission of this thesis.

3.4 Conclusions & Future Directions

The overall goal of performing this TnSeq study was to identify metabolites produced by alfalfa or metabolites inherent to the sprout germination environment that can be targeted for consumption by biocontrol agents to limit the growth of *Salmonella*. However, the progress made during this study was limited to achieving three of the six established objectives. A Tn5 transposon mutant library of 21,428 *Salmonella* Typhimurium 4/74 mutants was constructed, the library was successfully passaged through alfalfa sprouts over a six day time span and the cells were extracted from the sprout surface at the day 0, 1, 3, and 6 time points. Then, the genomic DNA of the extracted *Salmonella* Tn5 mutants was extracted for a library preparation protocol adapted from Karash et al.. Unfortunately, the protocol implemented to prepare a TnSeq amplicon library for each day's sample was insufficient due to potential errors such as PCR bias during the linear extension reaction and bottlenecks introduced during clean-up steps that may have caused overrepresentation of amplicons present in high abundance due to the initial PCR bias introduced to each sample at the linear extension step.

Despite these shortcomings, genomic DNA samples for each time point were submitted to Fasteris SA and this third-party laboratory service was contracted to perform a TnSeq library preparation and subsequent Illumina sequencing. However, the results were not available prior to the submission of this thesis for review, and the results were not included. Once the sequencing results are available from Fasteris SA, the sequencing files can be trimmed and aligned to the *Salmonella* Typhimurium 4/74 reference genome, then a quality check can be performed and the sequencing quality can be compared to what was observed with the protocol adapted from Karash et al. and the resulting Genewiz Illumina

sequencing results. If the results provided by Fasteris SA pass the quality check, the relative abundance of each transposon mutant for each time point can be determined. Then, a TnSeq application available through either Kbase, Galaxy, or BV-BRC can be used to determine the log change in relative abundance of each mutant at the 1, 3, and 6 day time points when compared to the initial abundance at day 0. From these results, mutants that experienced a negative log change in relative abundance would be identified, and the function of the gene containing the transposon insertion can be identified as conditionally essential towards the colonization and infection of alfalfa sprouts by *Salmonella* Typhimurium 4/74. Finally, genes that were identified as conditionally essential could be selected for further study via a classical genetics approach to verify the results of the TnSeq study.

Although this study has yet to be completed, a clear roadmap has been framed to continue this research to complete the goal of identifying metabolites produced by alfalfa or metabolites inherent to the sprout germination environment that can be targeted for consumption by biocontrol agents to limit the growth of *Salmonella*.

Chapter 4: Conclusion

The consumption of sprouts has been rapidly gaining consumer popularity since the 1980's; however, sprouts pose a serious risk towards food safety due to their germination conditions that create an ideal environment for the growth of foodborne pathogens such as *Salmonella*. As a result, numerous outbreaks of salmonellosis have occurred in fresh sprouts causing hundreds of illnesses and several high-profile interventions by the FDA to mitigate the risk towards consumers. Currently, *Salmonella* contamination is prevented in sprouts through practices such as seed decontamination, sampling of irrigation water and harvested sprouts, and post-harvest interventions such as chemical rinses, irradiation, and ultraviolet light treatment. However, the FDA does not require an antimicrobial intervention during sprout germination when environmental conditions are the most optimal for *Salmonella* growth and proliferation. To fill this gap in the sprouting industry, biocontrol is an ideal intervention since sprout-associated bacterial species specifically adapted for growing on the sprout surface can be isolated and formulated into biocontrol cocktails to mitigate *Salmonella* contamination.

Therefore, the focus of this thesis was divided between two goals. The first goal was centered on isolating/characterizing plant-associated bacteria from alfalfa sprouts and subsequently formulating a cocktail of plant-associated bacteria that would function as a biocontrol agent to reduce *Salmonella* contamination on alfalfa sprouts. The second goal focussed on utilizing a functional genomics approach through transposon insertion sequencing to determine *Salmonella* gene essentiality to the colonization and infection of alfalfa sprouts. With the identification of essential metabolic genes, metabolites produced by alfalfa or metabolites inherent to the sprout germination environment can be targeted for the consumption of these metabolites by biocontrol agents to limit the growth of *Salmonella*. Therefore, the formulation of plant-associated bacteria into a biocontrol cocktail could be specifically tailored towards the consumption of metabolite targets identified by the transposon insertion sequencing study.

To achieve the first goal, a cocktail of plant-associated bacterial species isolated from either alfalfa seeds or sprouts was formulated based on each constituents' ability to enhance the production of alfalfa biomass. The resulting cocktail was composed of *Pantoea agglomerans* JV6, *Priestia megaterium* JV24, *Pseudomonas koreensis* RG1, *Pantoea agglomerans* RG18, and *Pseudomonas putida* KT-2440. With a complex community of plant-associated bacteria, the PAB cocktail was found to increase alfalfa biomass production by 25% when compared to uninoculated seeds while not causing a detriment to the overall shelf-life of harvested alfalfa sprouts. Furthermore, the PAB cocktail was able to reduce the ability of *Salmonella* to colonize the alfalfa sprout surface throughout the entire sprout germination process resulting in a 3.1 log reduction of *Salmonella* after six days of germination.

In relation to the second goal, the progress made was limited to achieving three of the six established objectives. A Tn5 transposon mutant library of 21,428 *Salmonella*

Typhimurium 4/74 mutants was constructed, the library was successfully passaged through alfalfa sprouts over a six day time span and the cells were extracted from the sprout surface at the day 0, 1, 3, and 6 time points. Then, the genomic DNA of the extracted *Salmonella* Tn5 mutants was extracted for a library preparation protocol adapted from Karash et al.. Unfortunately, the protocol implemented to prepare a TnSeq amplicon library for each day's sample was insufficient due to potential errors inherent to the adapted protocol such as PCR bias during the linear extension reaction and bottleneck effects introduced during clean-up steps that may have led to overrepresentation of amplicons present in high abundance due to the initial PCR bias introduced at the linear extension step. Despite these shortcomings, genomic DNA samples for each time point were submitted to Fasteris SA and this third-party laboratory service was contracted to perform a TnSeq library preparation and subsequent Illumina sequencing. However, the results were not available prior to the submission of this thesis for review, and the results were not included.

In summation, a cocktail of plant-associated bacterial species was formulated to have a significant beneficial effect on alfalfa biomass production and cause a significant reduction in the cell density of *Salmonella* after six days of germination. Additionally, a transposon insertion sequencing study was performed to determine potential metabolites produced by alfalfa or metabolites inherent to the sprout germination environment for consumption of these metabolites by biocontrol agents to limit the growth of *Salmonella*; however, the results were not available for analysis prior to the submission of this thesis. Upon reception of the transposon insertion sequencing results, potential target metabolites can be identified and the plant-associated bacterial cocktail can be further refined to specifically target metabolites essential for the growth of *Salmonella* on alfalfa sprouts.

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