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Efficacy of Plant-derived Antimicrobials in Reducing Foodborne Pathogens in Agricultural Soil and Their Effect on Soil Nutrients and Microbiome

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**Efficacy of Plant-derived Antimicrobials in Reducing Foodborne Pathogens in Agricultural
Soil and Their Effect on Soil Nutrients and Microbiome**

Samantha Marie Fancher

B.S., Cornell University, 2012

A Thesis Submitted in Partial Fulfillment of the

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APPROVAL PAGE

Master of Science Thesis

Efficacy of Plant-derived Antimicrobials in Reducing Foodborne Pathogens in Agricultural Soil
and Their Effect on Soil Nutrients and Microbiome

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BR	Beta (β) – resorcylic acid
CDC	Centers for Disease Control and Prevention
CR	Carvacrol
EPA	Environmental Protection Agency
FDA	Federal Drug Administration
GRAS	Generally Recognized as Safe
HHS	Health and Human Services
NRCS	Natural Resources Conservation Service
PBH	Produce for Better Health Foundation
PBS	Phosphate buffered saline
PDA	Plant derived antimicrobial
PGPB	Plant growth-promoting bacteria
SAS	Statistical Analysis Software
SEA	Soil extract agar
TSA	Tryptic soy agar
TSB	Tryptic soy broth
TY	Thymol
UConn	University of Connecticut
USDA	US Department of Agriculture
XLD	Xylose lysine deoxycholate agar

Abstract

Bacterial pathogens such as *Salmonella* and *Listeria monocytogenes*, which have been implicated in several outbreaks involving fresh produce, can contaminate agricultural soil via contaminated manure or irrigation water. The rate of produce-associated outbreaks has been rising in recent decades and therefore, the potential for pre-harvest contamination is becoming of increasing concern. Several plant-derived antimicrobials (PDAs) have well-documented antimicrobial effects against foodborne pathogens, including *Salmonella* and *L. monocytogenes*, and have the potential as an alternative strategy to reduce these pathogens in agricultural soil. However, to ensure the health of crops being grown, these compounds should not result in any detrimental changes to the soil pH, nutrients and microbiome. In this study three PDAs, carvacrol (CR), thymol (TY), and β -resorcylic acid (BR) were investigated for their efficacy in reducing *Salmonella* and *L. monocytogenes* in soil. In addition, their effect on the soil pH and nutrient levels as well as the soil microbiome was determined.

All three compounds were effective in reducing both *Salmonella* and *L. monocytogenes* in soil. Carvacrol (0.25 and 0.5%) and 0.5% TY reduced both pathogens to undetectable levels by day 1. By day 21 all of the concentrations of PDAs reduced *Salmonella* and *L. monocytogenes* to below 1 Log CFU/g. Neither CR, TY, nor BR resulted in changes to the soil nutrients outside of normal limits ($P < 0.05$). However, BR reduced the soil pH to slightly below recommended levels, which increased the available aluminum concentration. The addition of CR and TY as well as the addition of *Salmonella* did not result in detrimental changes to the soil microbiome. Notably Carvacrol and TY increased the beneficial bacteria *Bacillus* and improved the community composition in the presence of *Salmonella*, especially the nitrogen-fixing bacteria *Rhizobiales*.

Therefore carvacrol and TY have the potential as soil amendments to reduce *Salmonella* and *L. monocytogenes* in sandy loam agricultural soil. Further in-field trials are necessary to determine the environmental fate of PDAs and their effect on plant health.

Chapter I

Introduction

Fruits and vegetables constitute an important part of our diet, and their per capita consumption in the US has been steadily rising for the last two decades. However, fresh produce has been increasingly associated with foodborne disease outbreaks in the US, raising concerns on their safety. From 1998 to 2008, produce accounted for approximately 46% of foodborne illness, making it the leading cause when compared to other food groups such as meat, dairy, poultry products, and seafood (Painter et al., 2013). Since produce is often not cooked before it is consumed, humans are at a greater risk for foodborne illness from consuming contaminated produce. *Salmonella spp.* and *Listeria monocytogenes* are two significant foodborne pathogens that can contaminate produce. Nontyphoidal *Salmonella* is the leading bacterial cause of outbreaks associated with contaminated produce, especially leafy greens and sprouts (DeWaal and Bhuiya, 2007). Similarly, *L. monocytogenes* has also been implicated in a number of foodborne outbreaks, including cantaloupes, bean sprouts, and pre-packaged apples (CDC, 2012; CDC, 2015a; CDC, 2015b). Although the outbreaks of food-borne listeriosis is less common than that of *Salmonella*, *L. monocytogenes* is associated with a higher mortality rate, and is known to cause fetal loss in pregnant women (Scallan et al., 2011).

There are several pathways by which *Salmonella* and *L. monocytogenes* can contaminate produce at the pre-harvest level. *Salmonella* is often found in the intestinal tracts of animals, both domestic and wild (Fatica and Schneider, 2011; Guo et al., 2002; Islam et al., 2004). It can survive in water bodies that are used for irrigation or in manure that is used for fertilization of the field (Baudart et al., 2000; Fatica and Schneider, 2011; Ingram et al., 2008). *L. monocytogenes* can naturally be present in the soil and can also be found in animal feces (Farzan et al., 2010; Vivant et al., 2013). Thus contaminated irrigation water and improperly composted manure can potentially lead to pathogen persistence in agricultural soil, thereby causing produce

contamination. Therefore, it is critical to include strategies for reducing these pathogens in soil for improving the microbiological safety of fresh produce.

Phytochemicals are natural compounds produced by plants that have well documented antimicrobial properties. A wide variety of phytochemicals have been used since very early times as flavoring agents and preservatives in foods (Wollenweber, 1988). Among the various plant compounds, carvacrol (CR), thymol (TY), and β -resorcylic acid (BR; 2,4-dihydroxybenzoic acid) have been shown to be effective against a number of foodborne pathogens, including *Salmonella* and *L. monocytogenes* (Adam et al., 1998; Blumenthal et al., 2000; Chun et al., 2005; Mattson et al., 2011; Upadhyay et al., 2014). Carvacrol and TY are extracted from oregano oil (*Origanum vulgare* (Lamiaceae)) and BR is a phytophenolic compound found in angiosperms (Bolton et al., 1986; Burt, 2004; Friedman et al., 2003). All three phytochemicals are classified as GRAS (generally recognized as safe) by the Food and Drug Administration (FDA, 2015; Friedman et al., 2003). Although composting strategies and the addition of plant essential oils to manure for controlling pathogens has been explored (Millner, 2009; Wells et al., 2014), their use as a soil amendment to reduce pathogens in agricultural fields has not been extensively investigated. However, it is critical that any soil amendment used in agriculture must support plant health by maintaining proper soil pH and nutrient level as well as a balanced endogenous microflora.

The objectives of this thesis were to: 1) investigate the efficacy of CR, TY, and BR in reducing *Salmonella* and *L. monocytogenes* in agricultural soil and 2) determine the effect of CR, TY, and BR on soil pH, nutrients and microbiome.

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Chapter II

Review of Literature

1. Fresh produce as a cause of foodborne illness:

The Centers for Disease Control and Prevention (CDC) estimates that 48 million Americans suffer from foodborne illnesses each year, with 128,000 hospitalizations, and 3,000 deaths (CDC, 2014a). The 14 most significant foodborne pathogens, which cause about 95% of foodborne illnesses, result in approximately \$14.6 to \$16.3 billion in economic losses per year (Anekwe and Hoffman, 2013; Hoffman et al., 2012; Scharff, 2012). From 1998 to 2008, Painter et al. (2013) determined that fresh produce was the leading cause of foodborne illnesses, accounting for approximately 46% of infections when compared to meat, dairy, poultry products, and seafood. This study also found that produce was responsible for 27% of illnesses caused by bacterial agents, preceded only by meat and poultry. Since fruits and vegetables are often not cooked before consumption, humans are more at risk for foodborne illnesses due to ingestion of contaminated produce. In addition, produce-associated outbreaks have highlighted a pattern of increasing rates in recent decades, from 0.7% of total outbreaks in the 1970s to 6% in the 1990s, and again to 13% from 1990 to 2005 (DeWaal and Bhuiya, 2007; Doyle and Erickson, 2008). On average, each American consumes 126.8 lbs of fresh fruit and 201.7 lbs of fresh vegetables per year (USDA, 2002). Due to multiple health benefits of eating fresh produce, dietary guidelines recommend that each person eat more fruits and vegetables, thereby resulting in an increased demand for fresh produce (HHS and USDA, 2010; PBH, 2015), which emphasizes the importance of improved safety of these products.

There are several factors that potentially contribute to the rise in produce-associated outbreaks. The increased consumption of produce combined with improved monitoring of outbreaks possibly plays a role (Doyle and Erickson, 2008). Fatica and Schnieder (2011) discussed several factors during production that could contribute to the rise in produce-associated outbreaks.

These researchers stated that intensive production of produce is a concern because in this system, products from many different farms may be pooled together in the processing facility, thereby getting exposed to a greater amount of handling, leading to contamination of a larger quantity of products. Similarly the globalization of food supply resulting in increased numbers of foods imported to the U.S., enhances the risk of possible contamination of these products. Finally, there is greater access to ready-to-eat products in which many different products may be cut or otherwise processed and mixed together. Cutting of fruits and vegetables has been attributed to make the products more susceptible to foodborne contamination because of the damage to the cells and mixing of many different products together.

2. *Salmonella* and *Listeria monocytogenes* in produce:

Salmonella spp. and *Listeria monocytogenes* are two of the most significant foodborne pathogens in the United States. *Salmonella* is a Gram-negative, facultatively anaerobic, flagellated, bacillus (Gianella, 2006). It is divided into two species, namely *S. bongori* and *S. enterica* (Fatica and Schneider, 2011). *S. enterica* contains over 2,500 serovars (Fatica and Schneider, 2011). Nontyphoidal *Salmonella* is a leading bacterial cause of foodborne illness in the US, responsible for an estimated 1.2 million illnesses, 9,000 hospitalizations, and 380 deaths per year (Scallan et al., 2011). It also has the greatest economic impact, resulting in approximately a \$3.3 billion loss per year (Hoffman et al., 2012). The CDC (2015a) describes salmonellosis as being characterized by symptoms, including diarrhea, fever, and abdominal cramps that last for 4 to 7 days. The invasion of host intestinal epithelial cells and production of toxins by *Salmonella* causes an inflammatory response resulting in diarrhea (Gianella, 1996). The treatment for moderate cases of salmonellosis involves the administration of oral fluids, although most people recover without the need for a treatment (CDC, 2015a). Intravenous fluids or antibiotics may be

used in serious cases. However, antibiotics are not recommended for treating milder cases because they do not shorten the time of illness, but extend the amount of time the bacteria are excreted, and can potentially lead to the development of bacterial antibiotic resistance (CDC, 2015a; Gianella, 1996).

Salmonella is the leading bacterial cause of outbreaks associated fruits and vegetables, accounting for about 18%, and is most commonly associated with leafy greens and sprouts (DeWaal and Bhuiya, 2007). Recently, there were three major outbreaks involving *S. Braenderup* in nut butter, *S. Enteritidis* in bean sprouts, and *S. Newport* in cucumbers that sickened 6, 115, and 275 people, respectively (CDC, 2014b; CDC, 2015b; CDC, 2015c). In 2005, Greene and coworkers described an outbreak of *S. Newport* associated with the consumption of tomatoes that affected 72 people. An identical strain had caused an outbreak three years prior, in 2002, which resulted in the illness of 510 people. In 2005, this *Salmonella* strain was actually traced back to pond water that was used to irrigate the tomato fields. This case highlights the potential for pre-harvest contamination of produce, and the fact that contamination in the environment can persist for extended periods of time (Greene et al., 2008).

L. monocytogenes is a Gram-positive, facultatively anaerobic, rod-shaped bacterium (Farber and Peterkin, 1991; Lawley, 2013). It has peritricous flagella, which gives it a tumbling motility between the temperatures 20 and 25°C (Farber and Peterkin, 1991). It is also psychrotrophic, meaning it can grow at refrigeration temperatures, thereby representing a significant risk to consumers (Lawley, 2013). *L. monocytogenes* is estimated to cause 1,600 illnesses, 1,500 hospitalizations, and 260 deaths per year with an economic loss amounting to \$2.6 billion (Hoffman et al., 2012; Scallan et al., 2011). Although *L. monocytogenes* is responsible for relatively fewer illnesses compared to *Salmonella*, there is approximately a 20% mortality rate in

those who develop listeriosis, making it responsible for about 19% of foodborne illness related deaths (CDC, 2013a; Scallan et al., 2011). *L. monocytogenes* is able to invade intestinal epithelial cells, primarily through Peyer's Patches and disperse through the blood and lymphatic systems (Marco et al., 1992). The symptoms and prognosis of the disease vary depending on the characteristics of the host. The elderly and immunocompromised persons usually experience septicemia and meningitis; pregnant women experience fever, fatigue, and aches as well as fetal loss, bacteremia or meningitis in their newborns, while healthy individuals may have gastroenteritis or be asymptomatic (CDC, 2013b; Dalton et al., 1997; Jackson et al., 2010; Painter and Slutsker, 2007; Riedo et al., 1994; Silk, 2012). Listeriosis is treated with antibiotics, most commonly penicillin, ampicillin, or amoxicillin alone or in combination with gentamicin (Allerberger and Wagner, 2010; CDC, 2013c; Hof, 2003). However, β -lactams are generally only bacteriostatic against *L. monocytogenes* and the use of gentamicin is not recommended for pregnant women (Allerberger and Wagner, 2010; Hof, 2003).

L. monocytogenes has been implicated in several severe outbreaks associated with fresh produce. For example, in 2011 cantaloupes produced from Jensen Farms in Colorado were linked to a nationwide outbreak of listeriosis with 147 persons becoming ill, in addition to 33 deaths and one miscarriage (CDC, 2012). In August of 2014, Wholesome Soy Products, Inc. initiated a recall of mung bean sprouts due to possible *L. monocytogenes* contamination. Five illnesses and two deaths were reported with this outbreak (CDC, 2015d). Similarly, in January of 2015 Granny Smith and Gala apples used to make prepackaged caramel apples were recalled because of contamination with *L. monocytogenes*. There were 35 illnesses reported with this outbreak and seven deaths, including one fetal loss (CDC, 2015e).

3. Potential sources of produce contamination with *Salmonella* and *L. monocytogenes*:

There are several pre-harvest factors that can lead to contamination of produce with pathogens, including *Salmonella* or *L. monocytogenes*. *Salmonella* is commonly found in the intestinal tract of food production and wild animals, including cattle, pigs, poultry and deer (Fatica and Schneider, 2011; Guo et al., 2002; Islam et al., 2004). *Salmonella* has been isolated from 31.5% of 359 samples from swine manure and fresh feces on 24 out of 31 farms tested (Farzan et al., 2010). It has also been found in 7.3% of 3,709 dairy cow fecal samples on 30.9% of 97 herds tested (Blau et al., 2005). This indicates the potential for transfer of *Salmonella* from feces to surface water subsequently used for irrigation of fields. It can also survive in manure that is used for fertilization of produce fields. It was found that *Salmonella* can survive for up to 184 days in manure and 332 days in manure-amended soil (You et al., 2006). Another study indicated that the length of time of pathogen survival could depend on the type of manure used (Hutchison, 2005). In fresh manure from different animal species, it was reported that *Salmonella* could be cultured from samples after 42 days in dairy cattle manure, 63 days in beef cattle manure, 32 days in pig manure, 63 days in poultry manure, and 16 days in sheep manure. In addition, *Salmonella* was found to survive in dirty water for up to 42 days. Since *Salmonella* can survive in water and water sediment, flooding fields can pose an additional risk of produce contamination (Baudart et al., 2000; Fatica and Schneider, 2011). Similarly, improper composting of manure can lead to pathogen survival and potential contamination of produce. For example, Ingram et al. (2008) reported that 53 and 7% of 15 commercially available compost facilities in the US had products that exceeded the limits for fecal coliforms and *Salmonella*, respectively.

Likewise, *L. monocytogenes* is ubiquitous in the environment, especially soil and water (Vivant et al., 2013). *L. monocytogenes* is carried by a variety of farm animals, including cattle, sheep, and goats (Brackett, 1998), thereby acting as a potential source for contamination of the

environment and food supply, including fresh produce. Studies have reported that 2 to 16% of healthy cattle carry this pathogen in the gastrointestinal tract, and excrete it into the farm environment (Seeliger, 1961; Skovgaard and Morgen, 1988). *L. monocytogenes* rarely causes disease in cattle, and animals can shed the pathogen in feces for several months or years (Lovett et al., 1987). Van Renterghem and coworkers (1991) found that *L. monocytogenes* was present in 20% of fresh cattle feces samples, and 5% of ground water samples tested. Further, *L. monocytogenes* being a robust, environmental pathogen, can survive in soil, water, and the environment for extended periods of time, thereby increasing the potential for foodborne transmission.

With regards to produce farms, *Salmonella* was present in 6% of water and 4.3% of sediment samples of irrigation systems on leafy green vegetable farms (Benjamin et al., 2013). In a survey of produce farms in New York, *Salmonella* was detected in 4.6% of samples, including water, soil, feces, and drag swabs, while *L. monocytogenes* was found in 15.0% of samples (Strawn et al., 2013a). The study reported that both bacteria were most likely to be isolated from water samples. In a similar study, manure application within one year and soil cultivation within seven days were associated with a higher likelihood of having a *Salmonella*-positive field while irrigation (within three days of sample collection), wildlife sightings (within three days of soil collection), and soil cultivation (within seven days before soil collection) were all associated with greater odds of a *L. monocytogenes* positive field (Strawn et al., 2013b).

4. Conventional approaches for controlling foodborne pathogens in produce:

For controlling pathogens such as *Salmonella* and *L. monocytogenes* in fresh produce, the industry generally relies on good agricultural practices (GAP) at the pre-harvest level and good handling practices (GHP) and antimicrobial treatments at the post-harvest level. GAP and GHP

follow the recommendations made in the FDA's "Guide to Minimize Microbial Food Safety Hazards for Fresh Fruits and Vegetables" (Food Safety Initiative Staff, 1997). Good agricultural practices include such recommendations as using irrigation water from reliable sources, composting manure properly, and monitoring environmental activity, especially the presence of wildlife and flooding, whereas GHP focuses on worker health and hygiene as well as facility sanitization.

Since manure can transmit pathogens to foods, several strategies for reducing pathogen carriage in food animals through the use of antibiotics, antimicrobials, probiotics, vaccines, and bacteriophages have been explored (Brabban et al., 2004). In addition, methods for reducing foodborne pathogens in manure, such as alternative methods of composting and the addition of plant essential oils have been investigated (Millner, 2009; Wells et al., 2014). However, the use of any kind of soil amendment or other application in the field for reducing pathogens has not been extensively investigated and is not a common practice in current food production.

5. Natural and environmentally friendly plant compounds as an alternative strategy for reducing foodborne pathogens in agricultural soil:

Plant-derived essential oils are a group of natural molecules that have been traditionally used as dietary constituents (Wollenweber, 1988), especially to preserve foods and enhance food flavor. Plant compounds have also been an important ingredient of many traditional medicines, and recent studies have investigated various beneficial properties of multiple essential oils, which range from antiseptic, anti-inflammatory, antioxidant and antimicrobial effects (Alma et al., 2003; Baratta et al., 1998; Deans and Ritchie, 1987). The plant-derived compounds used in this study include carvacrol (CR), thymol (TY), and β -resorcylic acid (BR). Carvacrol and TY are principal

ingredients in oregano oil obtained from *Origanum vulgare* (*Lamiaceae*), a common herb found in Europe and the Mediterranean. The essential oil obtained from *O. vulgare* has been found to be effective against bacterial and fungal infections of the gastrointestinal and genitourinary tract (Adam et al., 1998; Blumenthal et al., 2000; Chun et al., 2005). β -resorcylic acid (2, 4 dihydroxy benzoic acid) is a phytophenolic compound widely distributed among angiosperms (Bolton et al., 1986), and is a secondary metabolite that plays a key role in the biochemistry and physiology of plants (Friedman et al., 2003). β -resorcylic acid was effective in reducing *Salmonella* on tomatoes (Mattson et al., 2011) and *L. monocytogenes* on cantaloupe rinds (Upadhyay et al., 2014). All of these molecules are classified by the United States Food and Drug Administration as GRAS (generally recognized as safe) (FDA, 2015; Friedman, 2003). Although these compounds have been shown to inhibit various foodborne pathogens *in vitro* and in animals, studies determining their potential use in reducing pathogens in soil are limited. The only exception to this is that 0.5% cinnamaldehyde, extracted from cinnamon was effective in reducing *Escherichia coli* O157:H7 and *Salmonella* in organic soil while 0.5% eugenol, an ingredient in cloves, was found effective in reducing *Salmonella* (Yossa et al., 2010; Yossa et al., 2011). However, no information exists on the effect of these plant compounds on soil natural microflora. Thus, in light of their well-documented antimicrobial properties, CR, TY, and BR could potentially be used as a treatment for agricultural soil to control contamination of produce. Evidence also indicates that plant compounds are biodegradable and would not persist in the environment for extended periods of time to exert any negative effects (Heider and Fuchs, 1997; Isman, 2006). Additionally, at present there are no known reports of pathogens developing resistance to such antimicrobial plant compounds (Ohno et al., 2003; Domadia et al., 2007), since they contain several functional groups in their structure, and target bacterial cells by multiple mechanisms (Burt, 2004).

6. Soil pH, nutrients, and microbiome - important conditions in maintaining plant health:

Soil composition, primarily the pH, nitrogen content, organic matter, macronutrients, and micronutrients are important for maintaining plant health (Johnston, 2011). The optimal conditions for growing most produce in the northeastern US include a pH between 6 and 6.8, 2,016 to 2,687 kg/ha of calcium, 196 to 279 kg/ha of magnesium, 16 to 22 kg/ha of phosphorus, and 280 to 391 kg/ha of potassium (UConn Soil Nutrient Analysis Laboratory, 2004).

The diversity of endogenous bacteria found in soil also plays a key role in the health of the plants. Bacteria make up the majority of the soil microbiota with an estimated 60,000 different species of bacteria that reside mostly in the top 10 cm of soil where the organic matter is present (Reid and Wong, 2005; Sylvia et al., 1998; Zuberer, n.d.). Janssen (2006) identified the most common phyla of soil bacteria by analyzing bacterial libraries from different types of soil, which include *Proteobacteria*, *Acidobacteria*, *Actinobacteria*, *Verrucomicrobia*, *Bacteroidetes*, *Chloroflexi*, *Planctomycetes*, *Gemmatimonadetes*, and *Firmicutes*, together making up about 92% of soil libraries. *Proteobacteria* and *Acidobacteria* appeared to be the two most common phyla, as all of the libraries analyzed had some sequences belonging to these phyla. However, the range of bacteria that are present depends on the type of soil, type of plants grown, and the location in proximity to the root system (Marschner, 2001). It is estimated that only about 10% of soil bacteria can be cultured in the laboratory, and it is therefore necessary to use molecular techniques to evaluate the full diversity (Janssen, 2006).

Bacteria present in the rhizosphere, the layer of soil that directly interacts with the plant's root system, plays an intricate role in the health of the plant. Compant et al. (2010) reviewed how bacteria are attracted to the root system by chemotaxis due to root exudates which include various carbohydrates, amino acids, organic acids, and other substances. They reported how some bacteria

present in the rhizosphere may have a negative effect on the plant, while some may have no obvious effect at all. On the other hand, many support the growth and health of plants, and may even aid in reducing pathogen infection. These are referred to as plant growth-promoting bacteria (PGPB) (Compant et al., 2010). Raaijmakers et al. (2009) reported that some PGPB are capable of inhibiting pathogen growth or attachment to the plant by competition or antagonism. Competition refers to the competition for space and nutrients in the soil that results in reducing pathogenic bacteria. Antagonism generally occurs when PGPB produce secondary metabolites and lytic enzymes that have an inhibitory effect on pathogenic organisms. For example, *Pseudomonas fluorescens* produces hydrogen cyanide and a secondary metabolite known as DAPG, which suppresses *Thielaviopsis basicola*, a fungus that causes black root rot of tobacco (Keel et al., 1992; Raaijmakers et al., 2009; Voisard et al., 1989). Some PGPB, such as pseudomonads, also secrete effectors through a type III secretion system, which can directly decrease virulence in pathogens, but much about the mechanism by which this occurs is unknown (Raaijmakers et al., 2009). Besides suppressing plant pathogens, some bacteria can directly benefit the plants. One example of this is *P. fluorescens* which can induce plants to produce chemicals that are associated with defending pathogens (Pieterse et al., 2003; Raaijmakers et al., 2009).

Another important role of PGPB is the cycling of nutrients such as carbon, nitrogen, phosphorous, sulfur, manganese, iron, and phosphate (Osorio Vega, 2007; Zuberer, n.d.). The bacteria metabolize these nutrients into a form that is available for uptake by plants. One of the most well studied interactions is the ability of some bacteria to fix N₂ into organic forms that can be used by plants (Osorio Vega, 2007). Some of these genera include *Rhizobium*, *Bradyrhizobium*, *Allorhizobium*, *Sinorhizobium*, and *Mesorhizobium* (Osorio Vega, 2007). In general *Proteobacteria*, such as *Pseudomonas* and *Burkholderia*, and *Firmicutes* such as *Bacillus*

and related genera, have been well-documented as beneficial soil bacteria (Raaijmakers et al., 2009).

Since the diversity of endogenous soil bacteria plays an intricate role in the health of the soil and consequently the plant, it is important for any soil amendment to maintain this balance. Experiments indicate that plant compounds generally exert a selective killing on pathogens as compared to commensal bacteria in animals (Hawrelak et al., 2009; Thapa et al., 2012). The reasons for this are not fully understood, but it is hypothesized that since these compounds are naturally present in the environment and have evolved with the surrounding organisms, the commensal bacteria are not targeted (Hawrelak et al., 2009; Thapa et al., 2012).

7. Hypothesis and objectives:

Based on the published research, it was hypothesized that the CR, TY, and BR exert a significant antimicrobial effect on *Salmonella* and *L. monocytogenes* in agricultural soil with minimal detrimental effects on the soil composition and microbiome. Therefore, the specific objectives of this study were:

- 1) To investigate the efficacy of CR, TY, and BR in reducing *Salmonella* and *L. monocytogenes* in agricultural soil.
- 2) To determine the effect of CR, TY, and BR on soil pH, nutrients and microbiome.

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Chapter III

Efficacy of plant derived antimicrobials in reducing *Salmonella* and *Listeria monocytogenes* in agricultural soil

Abstract

Agricultural soil is a potential source of contamination for foodborne pathogens, such as *Salmonella* and *Listeria monocytogenes*, to fresh produce. Pre-harvest contamination of fresh produce is generally minimized through good agricultural practices (GAP), but these strategies are not completely effective in rendering the produce safe for human consumption. This study aimed to develop environmentally friendly soil amendments using natural, FDA approved plant derived antimicrobials (PDAs), namely carvacrol (CR), thymol (TY) and beta-resorcylic acid (BR) for reducing *Salmonella* and *L. monocytogenes*.

Ten gram portions of sandy loam soil collected from a commercial produce farm was inoculated with either a four serovar mixture of *Salmonella* (*S. Montevideo*, *S. Braenderup*, *S. Baildon*, and *S. Newport*) or a four strain mixture of *L. monocytogenes* at ~ 6 Log CFU/g, followed by the addition of CR, TY, and BR at 0.25 and 0.5%. The treated soil samples were stored at room temperature for 21 days, and analyzed on days 0, 1, 3, 7, 14, and 21 for *Salmonella* or *L. monocytogenes*. The effect of the PDAs on the total endogenous soil bacteria was also determined.

On day 1, CR at 0.25 and 0.5%, and TY at 0.5% reduced *Salmonella* and *L. monocytogenes* in soil to undetectable levels ($P < 0.05$). *Salmonella* population was decreased by ~ 5.3, 2.7, and 0.9 Log CFU/g in soil containing 0.25% TY, 0.5% BR, and 0.25% BR, respectively by day 1 ($P < 0.05$). Thymol at 0.25%, and BR at 0.5% and 0.25% reduced *L. monocytogenes* by 6.1, 2.8, and 1.4 Log CFU/g, respectively on day 1 ($P < 0.05$). On day 21, all PDA treatments reduced *Salmonella* and *L. monocytogenes* to < 1 Log CFU/g ($P < 0.05$). However, approximately 3 log CFU/g of *Salmonella* and *L. monocytogenes* was recovered from control soil samples. Furthermore, the PDA treatments had no significant effect on the counts of endogenous soil bacteria ($P > 0.05$). This study highlights the potential of CR, TY, and BR as environment friendly

soil amendments to reduce *Salmonella* and *L. monocytogenes* in soil, thereby decreasing the risk of produce contamination. However, follow-up studies under field conditions are warranted.

1. Introduction

Fresh produce is currently the leading cause of foodborne illness when compared to other food products, and the number of produce associated cases has been increasing since 1970 (DeWaal and Bhuiya, 2007; Doyle and Erickson, 2008; Painter et al., 2013). The Center for Science in the Public Interest database of foodborne illnesses reported that 28,315 of 138,622 cases (20.4%) or 554 of 4486 outbreaks (12.3%) in the United States from 1990 through 2003 were associated with the consumption of fresh produce (Dewaal et al., 2006). A recent report from the US Centers for Disease Control and Prevention indicated that approximately 46% of all foodborne illnesses are caused by contaminated produce (Painter et al., 2013). The increase in the per capita consumption of fruits and vegetables contributes partly to this, besides the improved monitoring of outbreaks (Doyle and Erickson, 2008; Fatica and Schneider, 2011; USDA, 2002). In addition, the intensive production of fresh produce, where products from many different farms may be pooled together in a single processing facility, and the large number of produce types imported to the US have attributed to the rise in produce-associated outbreaks (Doyle and Erickson, 2008; Fatica and Schneider, 2011).

Several pre-harvest factors potentially contribute to fresh produce contamination with pathogenic microorganisms. These include the proximity or contact with soil, feces, use of improperly composted manure, contaminated irrigation water, wild and domestic carrier animals and farm workers (Beuchat, 1996, 2002; Harris et al., 2003). Among the various foodborne pathogens, *Salmonella* spp. and *Listeria monocytogenes* are two of the major ones associated with produce contamination (Beuchat, 2002; DeWaal and Bhuiya, 2007). *Salmonella* spp. have been reported to cause 18% of produce-associated outbreaks, making it the leading bacterial cause of foodborne illnesses linked to produce consumption (DeWaal and Bhuiya, 2007). Similarly, *L.*

monocytogenes has been responsible for several outbreaks involving fresh produce (CDC, 2015a,b). The most severe outbreak occurred in 2011 when *L. monocytogenes* on cantaloupes from Jensen Farms in Colorado infected 147 people, causing 33 deaths and one miscarriage (CDC, 2012).

A study on produce farms in New York reported that *Salmonella* was present in 4.6% of water, soil, feces, and drag swab samples, while *L. monocytogenes* was found in 15% of the samples (Strawn et al., 2013). These pathogens can be transferred to soil from contaminated manure or irrigation water (Beuchat, 2002; Fatica and Schneider, 2011). Therefore reducing their persistence in the soil could potentially decrease produce contamination. However, the use of any soil amendment or other application in the field for reducing pathogens in agricultural soil has not been extensively investigated.

Plants are capable of synthesizing a large number of molecules (Geissman, 1963), most of which are produced as a defense mechanism against predation by microorganisms and insects. A variety of plant-derived compounds are active components in traditional medicines (Wollenweber, 1988), and several compounds have demonstrated antimicrobial activity (Burt, 2004; Holley and Patel, 2005). Carvacrol (CR) and thymol (TY) are phytochemicals extracted from oregano oil, whereas β -resorcylic acid (BR) is a phenolic compound commonly found in angiosperms (Bolton et al., 1986; Friedman et al., 2003). All three of these phytochemicals are classified as generally recognized as safe (GRAS) by the Food and Drug Administration (FDA, 2015; Friedman, 2003). Our previous research revealed that these compounds are effective in reducing *Salmonella* on tomatoes (Mattson et al., 2011), *L. monocytogenes* on cantaloupe rinds (Upadhyay et al., 2014), and *Escherichia coli* O157:H7 on apples (Baskaran et al., 2013). In addition, cinnamaldehyde, a key ingredient in cinnamon was found effective in reducing *Salmonella* and *E. coli* O157:H7,

while another phytochemical, eugenol was effective in reducing *Salmonella* in soil (Yossa et al., 2010, 2011). Based on this previous research the objective of this study was to determine the efficacy of CR, TY, and BR in reducing *Salmonella* and *L. monocytogenes* in agricultural soil.

2. Materials and Methods

2.1. Preparation of bacterial strains

The four serovars of *Salmonella enterica* used for this study included *S. Braenderup*, *S. Baildon*, *S. Newport*, and *S. Montevideo*, all of which were isolated from tomatoes. The four strains of *L. monocytogenes*, included ATCC Scott A and ATCC 19115, which were human isolates, 1 (apple isolate), and Presque-598 (source unknown). The strains of *Salmonella* were cultured separately in 10 ml of tryptic soy broth (TSB, Difco, Becton Dickinson, Sparks, MD) and incubated at 37°C for 24 h. After three successive transfers, equal volumes of the cultures were combined and sedimented by centrifugation (3,600 g for 15 min at 4°C). The pellet was washed twice and resuspended in 10 ml of phosphate buffered saline (PBS; pH 7.0), and used as the inoculum (8 Log CFU/ml). The bacterial count of the individual cultures and the four-serovar mixture were confirmed by plating 0.1-ml portions of appropriate dilutions on xylose lysine deoxycholate agar (XLD; Difco, Becton Dickinson) plates and incubating the plates at 37°C for 24 h. The strains of *L. monocytogenes* were pre-induced for resistance to 50 µg/ml of rifampicin (Sigma-Aldrich Corp, St. Louis, MO) for selective enumeration. They were grown separately in 10 ml of TSB with 0.6% yeast extract (Difco) and 50 µg/ml of rifampicin at 37°C for 18 h, and equal volumes of the cultures were combined. The combined culture was centrifuged at 3600 rpm for 15 min, pellet resuspended in 10 ml of PBS, and used as the inoculum (8 Log CFU/ml). The bacterial count of the individual cultures and the four-strain mixture was again confirmed, this time by plating appropriate dilutions on tryptic soy agar (TSA; Difco) plus 50 µg/ml of rifampicin.

2.2. Inoculation and treatments

Sandy loam soil was collected from a commercial produce farm by mixing equal portions from two separate locations in the field. The sampling locations had not been treated with any pesticides and the samples were collected during two different times during the growing season. Ten g of the soil was added to a sterile stomacher bag (Nasco, Fort Atkinson, Wisconsin) followed by the addition of 100 µl of the *Salmonella* or *L. monocytogenes* culture (~ 6 Log CFU/g). The soil samples were mixed by hand for 1 minute, and CR, TY or BR was added at 0.25 or 0.5% (w/w). The soil samples were again mixed by hand for 1 min, and stored at room temperature (23°C) to mimic the soil temperature in the northeastern US during the growing season (NRCS, 2015). The counts of *Salmonella*, *L. monocytogenes*, and the endogenous soil bacteria were determined on days 0, 1, 3, 7, 14, and 21 of storage.

2.3. Enumeration of *Salmonella*, *L. monocytogenes*, and endogenous soil bacteria

On each sampling day, each 10 g soil sample was added with 10 ml of Dey–Engley neutralizing broth (D/E neutralizing broth; Difco), and stomached at normal speed for 1 min. The samples were then serially diluted (1:10 in PBS) and 100 µl was plated on different media for bacterial enumeration. For the enumeration of *Salmonella*, the samples were plated on xylose lysine deoxycholate (XLD; Difco) agar, whereas for estimating the viable *L. monocytogenes* counts, dilutions were plated on TSA containing 50 µg/ml of rifampicin. For estimating the endogenous soil bacteria, the samples were plated on soil extract agar (SEA; HiMedia, Mumbai, India), a medium that has been shown to support the growth of a diversity of soil bacteria as compared to other conventional media (Lutton, E. et al., 2013; Prakesh, V. et al., 2007). The plates were incubated at 37°C for 24 before counting colonies.

2.4. Statistical analysis

The experiments were each set up as a completely randomized design with a two-way factorial. The factors included phytochemical concentration and time. All the treatments were done in duplicate and each experiment was repeated three times. The data were analyzed using the PROC-GLM procedure of SAS version 9.4 and significance was detected as $P < 0.05$.

3. Results

3.1. Effect of phytochemicals on *Salmonella* in soil

The effect of the 0.25 and 0.5% CR, TY, and BR on *Salmonella* is depicted in Fig. 1. On day 0, the average population of *Salmonella* in inoculated control soil samples was ~ 5.5 Log CFU/g. Most treatments significantly reduced *Salmonella* by day 1 when 0.25 and 0.5% CR and 0.5% TY decreased the pathogen to undetectable levels (enrichment negative) ($P < 0.05$). *Salmonella* was reduced by ~ 5.0 Log CFU/g and 2.7 Log CFU/g in the presence of 0.25% TY and 0.5% BR, respectively ($P < 0.05$). By day 3, *Salmonella* was reduced to undetectable levels by 0.25% TY ($P < 0.05$). All treatments reduced *Salmonella* to undetectable levels by day 21 ($P < 0.05$), except in 0.25% BR containing samples, where the pathogen count was < 1 Log CFU/g. However, more than 3 log CFU/g of *Salmonella* was recovered from control samples on day 21.

3.2. Effect of phytochemicals on *L. monocytogenes* in soil

All three compounds were also effective in reducing *L. monocytogenes* in soil. The effect of 0.25 and 0.5% CR, TY, and BR on *L. monocytogenes* is depicted in Fig. 2. After inoculation on day 0, the mean concentration of *L. monocytogenes* in soil was ~ 6.5 Log CFU/g. All the treatments significantly reduced *L. monocytogenes* by day 1, with 0.25 and 0.5% CR and 0.5% TY reducing it to undetectable levels (enrichment negative) ($P < 0.05$). In the treatments containing

0.25% TY and 0.25 and 0.5% BR, *L. monocytogenes* counts were decreased by ~ 6.0, 1.4, and 2.8 Log CFU/g, respectively ($P < 0.05$). Although 0.25% TY reduced *L. monocytogenes* to undetectable levels on days 7 and 14, samples on day 21 tested positive by enrichment ($P < 0.05$). By day 21, all of the other treatments also reduced the pathogen count to undetectable levels ($P < 0.05$).

3.3. Effect of PDAs on the endogenous soil bacteria

The concentration of endogenous soil bacteria ranged from ~ 6.2 to 6.5 Log CFU/g on day 0. None of the treatments resulted in a significant change in the bacterial population on any day of storage ($P > 0.05$), and their counts remained the same throughout the storage period (Fig. 3).

4. Discussion

Since *Salmonella* and *L. monocytogenes* can colonize food animals and are shed in their feces, treatment of soil with fresh or improperly composted manure can potentially contaminate agricultural soil, thereby leading to pathogen persistence in soil and subsequent produce contamination. In addition, the use of contaminated irrigation water could contribute to contamination of soil and root vegetables with pathogens (Islam, 2004a,b; You et al., 2006). Thus it is critical to include interventions targeting soil for controlling potential transfer of bacterial pathogens to produce.

This study demonstrated that three naturally occurring phytochemicals, CR, TY, and BR were effective in reducing both *Salmonella* and *L. monocytogenes* in sandy loam soil, a common soil type used for crop production in the northeastern US. Not only did all three of the phytochemicals significantly reduce *Salmonella* and *L. monocytogenes* by day 1, they continued to exert a bactericidal effect on the pathogens until 21 days of storage. Similar results were

reported by Yossa et al. (2011), who while determining the effect of plant essential oils on *Salmonella* observed that 0.5, 1, 1.5 and 2% of cinnamaldehyde, an ingredient in cinnamon oil, was effective in inactivating the pathogen in several types of organic soil. Another study from the same group of researchers (Yossa et al., 2010) found that cinnamaldehyde was also effective in killing significant populations of *E. coli* O157:H7 in organic soil. However, these researchers observed that eugenol, a phytochemical from clove oil did not reduce *E. coli* O157:H7 in soil compared to untreated samples.

Unlike the inhibitory effect on pathogens, our results indicate that CR, TY, and BR at all tested concentrations did not exert any deleterious effect on the total endogenous soil bacteria. This is advantageous since many endogenous soil bacteria are well-documented to exert beneficial effects on plant health (Compant et al., 2010). However, the reason behind the selective killing of pathogens is not clear although we observed similar results in our previous animal studies, where in-feed supplementation of trans-cinnamaldehyde to chickens reduced cecal populations of *S. Enteritidis* without causing any adverse effect on total cecal bacterial populations (Upadhyaya et al., 2015a) or the cecal microbiome (Upadhyaya et al. 2015b). It is hypothesized that since these compounds are naturally present in the environment and have evolved with the surrounding organisms, the commensal bacteria are not targeted (Hawrelak et al., 2009; Thapa et al., 2012). Nonetheless, further detailed studies on the long-term effect of these phytochemicals on various groups of endogenous soil bacteria are necessary.

Evidence suggests that phytochemicals are biodegradable, and would not persist in the environment for long periods of time to potentially impact the ecosystem (Heider and Fuchs, 1997; Isman, 2006). In addition, being natural and GRAS, CR, TY, and BR could be used in both organic and conventional agriculture. Since phytochemicals contain multiple chemical groups in their

structure, their antimicrobial activity is believed to be due to different mechanisms (Burt, 2004). Many essential oils or their components due to their hydrophobicity target the lipid containing bacterial cell membrane (Sikkema et al. 1994), making it permeable and leading to leakage of ions and other cell contents (Cox et al. 2006). Furthermore, some phytochemicals are reported to kill bacteria by inhibiting energy generation and glucose uptake (Gill and Holley, 2004). Thus because of their manifold antimicrobial mechanisms, the potential for bacteria for developing resistance to plant antimicrobials is believed to be smaller (Domadia et al. 2007; Ohno et al. 2003).

In summary, CR, TY, and BR were effective in reducing *Salmonella* and *L. monocytogenes* in sandy loam soil without affecting the concentration of endogenous soil bacteria. Our future studies will investigate the effect of these phytochemicals on soil nutrient composition and microbiome. In addition, long-term field studies are warranted before recommending their application in soil for improving produce safety.

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Fig. 1.

The effect of 0.25 and 0.5% carvacrol (CR), thymol (TY), and β -resorcylic acid (BR) on *Salmonella* in sandy loam agricultural soil. The error bars represent SEM (n=6). Ten g of sandy loam soil was inoculated with ~ 6.0 Log CFU/g of *Salmonella*. After homogenization the treatments were added at the respective concentrations. The samples were again homogenized and stored in the dark at room temperature. Surviving *Salmonella* was enumerated on days 0, 1, 3, 7, 14, and 21.

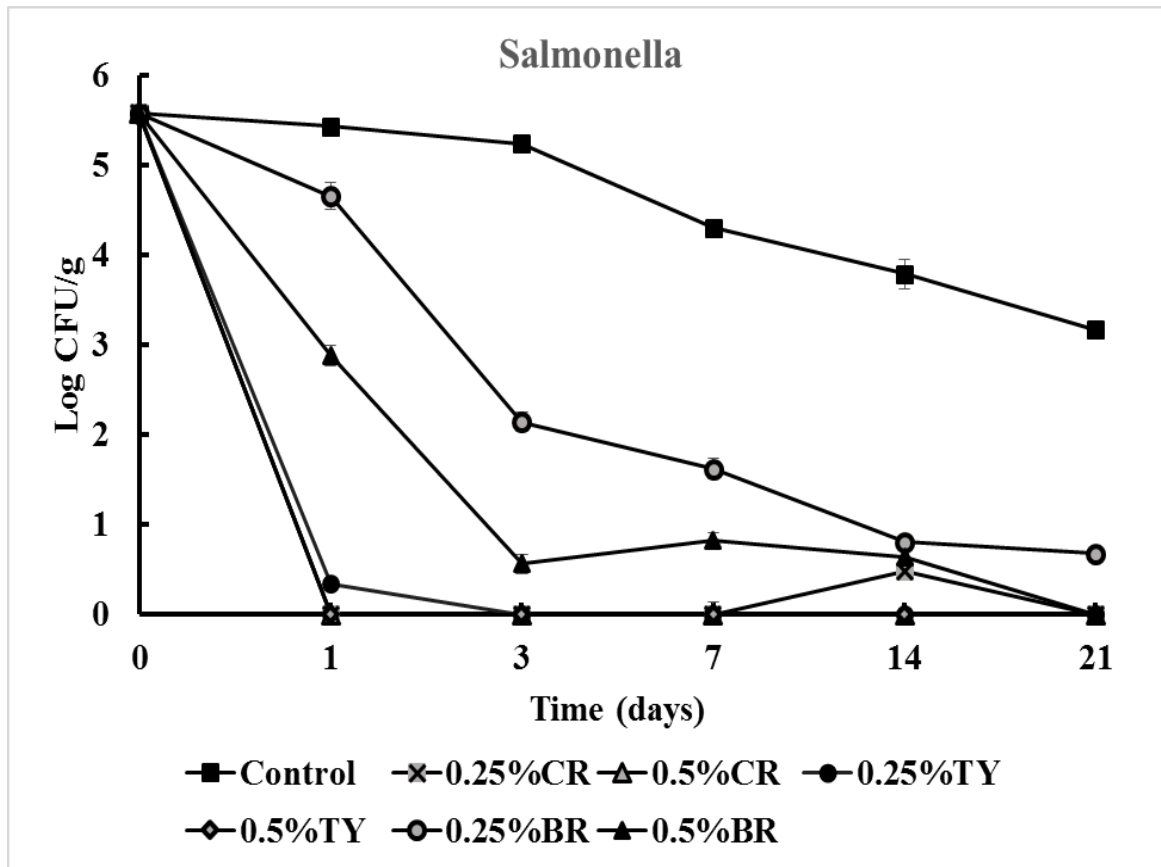


Fig. 2.

The effect of 0.25 and 0.5% carvacrol (CR), thymol (TY), and β -resorcylic acid (BR) on *L. monocytogenes* in sandy loam agricultural soil. The error bars represent SEM (n=6). Ten g of sandy loam soil was inoculated with ~ 6.0 Log CFU/g of *L. monocytogenes*. After homogenization the treatments were added at the respective concentrations. The samples were again homogenized and stored in the dark at room temperature. Surviving *L. monocytogenes* was enumerated on days 0, 1, 3, 7, 14, and 21.

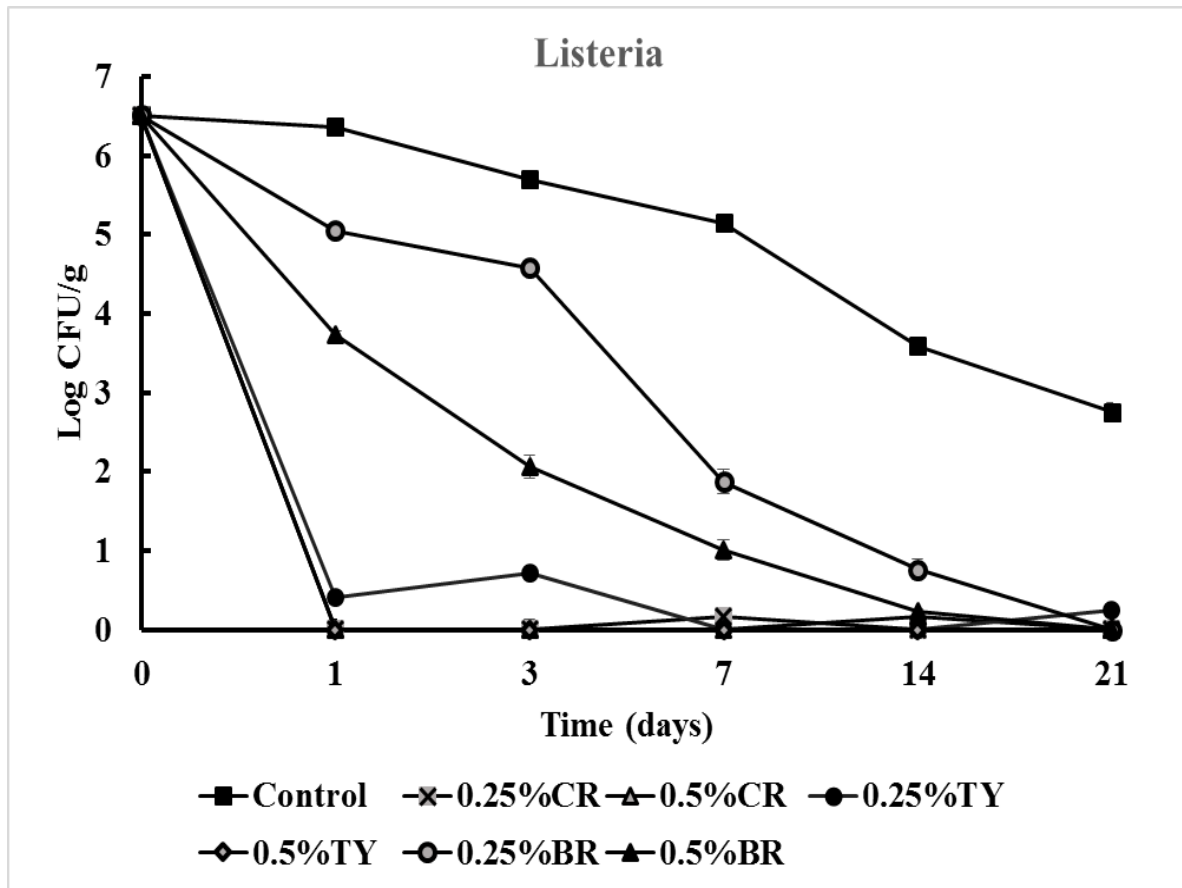
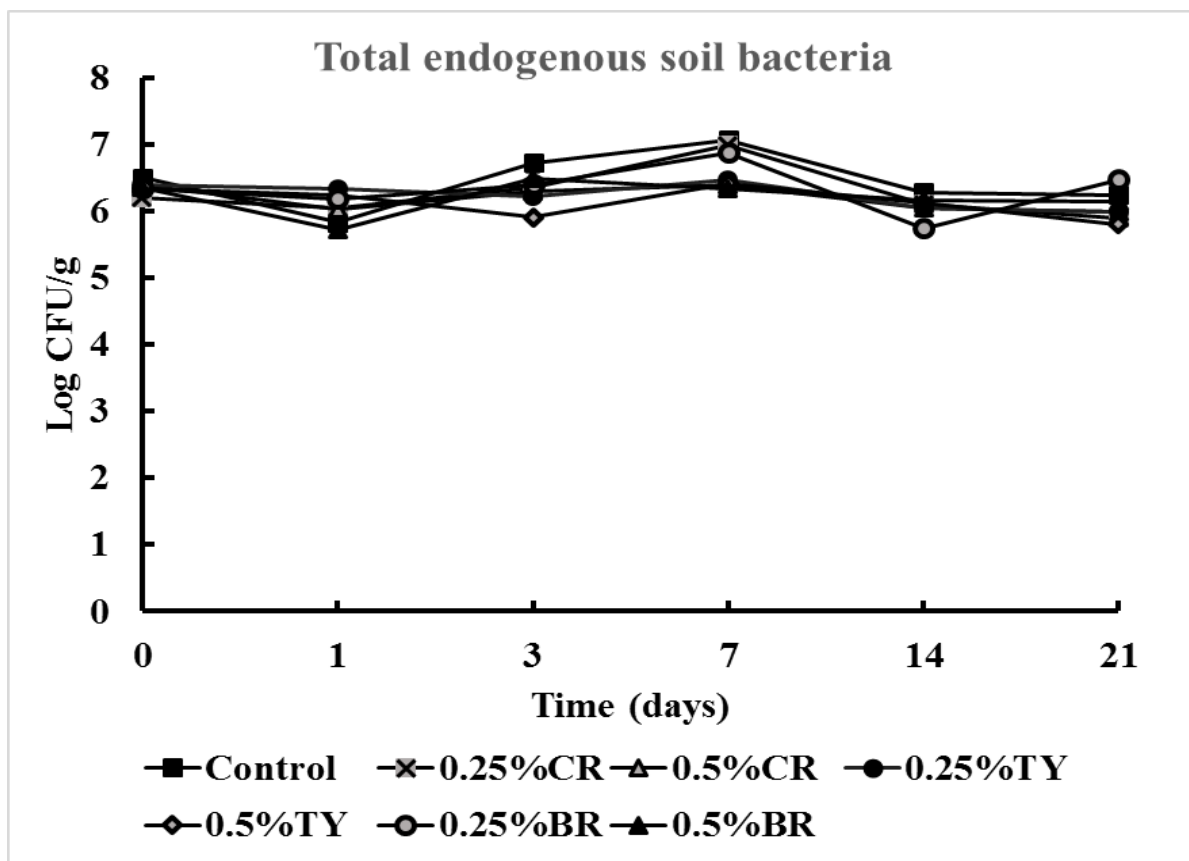


Fig. 3.

The effect of 0.25 and 0.5% carvacrol (CR), thymol (TY), and β -resorcylic acid (BR) on the endogenous soil bacteria in sandy loam agricultural soil. The error bars represent SEM (n=6). The treatments were added to the soil at the respective concentrations and homogenized. The samples were stored in the dark at room temperature and the endogenous soil bacteria was enumerated on days 0, 1, 3, 7, 14, and 21.



Chapter IV

The effect of plant derived antimicrobials on soil pH, nutrients levels and microbiome composition

Abstract

Agricultural soil represents a potential source for contamination of fresh produce with foodborne pathogens. Our previous studies revealed that three plant-derived antimicrobials (PDAs), carvacrol (CR), thymol (TY), and β -resorcylic acid (BR) exerted significant antimicrobial effect on *Salmonella* and *L. monocytogenes* in soil. However, for these PDAs to be considered beneficial soil amendments, they must maintain healthy soil conditions such as pH, nutrients, and the microbiome, especially because many bacteria have been shown to exert direct and indirect positive effects on plant health. Therefore, this study investigated the effect of CR, TY, and BR on soil pH, nutrients levels and the microbiome.

Sandy loam soil collected from a commercial produce farm was treated with 0.5% CR, TY, or BR and stored at room temperature for 21 days. On days 0, 7, 14, and 21, the soil samples were analyzed for changes in pH and nutrient content. To determine the effect of PDAs on the soil microbiome either in the presence or absence of *Salmonella*, soil aliquots (20 g) inoculated with or without ~ 6 Log CFU/g of a four serovar mixture of *Salmonella* (*S. Montevideo*, *S. Braenderupe*, *S. Baildon*, and *S. Newport*) were mixed with CR and TY (0.5%), and incubated as described earlier. On days 0, 7, 21, and 42, total microbial DNA from soil samples (0.5 g) were extracted, and subjected microbiome analysis.

None of the three plant compounds resulted in changes to the soil nutrient levels outside of normal limits ($P < 0.05$). However, BR reduced the soil pH to slightly below recommended levels ($P < 0.05$), which resulted in an elevated available aluminum concentration. The addition of CR and TY as well as *Salmonella* did not significantly change the alpha diversity of the soil microbial communities, although they affected the beta-diversity. However, there were no apparent detrimental changes to the major soil phyla. Carvacrol and TY increased the population of

beneficial soil bacteria, *Bacillus*, and improved the community composition when compared to *Salmonella* alone, especially by increasing *Rhizobiales* ($P < 0.05$). Results indicate that CR and TY did not exert any detrimental effect on soil nutrient and microbiome composition, thereby suggesting their potential use as soil amendments for improving the microbiological safety of fresh produce.

1. Introduction

Pre-harvest produce contamination by foodborne pathogens is a rising concern in the food industry as fruits and vegetables are the leading cause of foodborne outbreaks when compared to other food groups (DeWaal and Bhuiya, 2007; Doyle & Erickson, 2008; Painter et al., 2013). Several pathogens, including *Salmonella*, which is the leading bacterial cause of produce-associated outbreaks (DeWaal and Bhuiya, 2007), are excreted in the feces of domestic and wild animals. Therefore treatment of soil with fresh or improperly composted manure can potentially contaminate agricultural soil, subsequently causing produce contamination. Moreover, contaminated irrigation water could contribute to pathogen contamination of soil and root vegetables (Islam, 2004a,b; You et al., 2006). Thus pre-harvest interventions for reducing pathogens in agricultural soil are valuable for improving the microbiological safety of fresh produce. However, it is important that any soil amendment used in agriculture must support plant health by maintaining proper soil pH and nutrient level as well as a balanced endogenous microflora.

The three major soil nutrients critical for plant health are nitrogen, phosphorous, and potassium (Lines-Kelly, 2004). Nitrogen is necessary for plant growth because it forms proteins and chlorophyll. Phosphorous aids in early root development and photosynthesis, while potassium helps in disease resistance and moving nutrients through the plant (Lines-Kelly, 2004). In addition, calcium is used for root and leaf development, whereas magnesium is vital for the production of chlorophyll (Lines-Kelly, 2004). Boron, copper, iron, manganese and zinc are micronutrients and their levels are generally sufficient in soils as long as the pH and organic matter content of the soil are suitable (Lines-Kelly, 2004). Finally, the pH greatly influences the availability of nutrients in the soil (Lines-Kelly, 2004).

Studies indicate that several plant compounds exert a selective killing on pathogens as compared to commensal bacteria in animals (Hawrelak et al., 2009; Thapa et al., 2012). Although the reasons for this are not fully understood, it could be beneficial in this particular application since the diversity of bacteria in the soil is known to play an important role in the health of plants (Raaijmakers et al., 2009; Zuberer, n.d.). Bacteria make up the majority of soil microbiota with (Sylvia et al., 1998; Zuberer, n.d.) an estimated 60,000 different species (Reid and Wong, 2005). Overall, the most common phyla are *Proteobacteria*, *Acidobacteria*, *Actinobacteria*, *Verrucomicrobia*, *Bacteroidetes*, *Chloroflexi*, *Planctomycetes*, *Gemmatimonadetes*, and *Firmicutes* representing approximately 92% of soil libraries (Jannsen, 2006). However, the populations of bacteria can vary greatly depending on the type of soil, type of plant being grown, and location in proximity to the root system (Marschner, 2001). The diversity of bacteria in the soil are known to play an important role in the health of plants (Raaijmakers et al., 2009; Zuberer, n.d.), where beneficial soil bacteria, also referred to as plant growth-promoting bacteria (PGPB), support the growth of plants and aid in reducing pathogen infection through several mechanisms (Compant et al., 2010). Moreover, PGPB can exert direct effects on plants such as by inducing the plant immunity. In general, *Proteobacteria*, such as *Pseudomonas* and *Burkholderia*, and *Firmicutes* such as *Bacillus* and related genera, have been well-documented as beneficial soil bacteria (Raaijmakers et al., 2009). Furthermore, PGPB play an important role in utilizing nutrients such as carbon, nitrogen, phosphorous, sulfur, manganese, iron, and phosphate by metabolizing them into forms that can be taken up by the plant (Osorio Vega, 2007; Zuberer, n.d.). One such well-characterized function is the ability of bacteria in the order *Rhizobiales* to fix N₂ into organic forms that can be used by the plant for protein formation (Osorio Vega, 2007).

Previous research in our laboratory indicated that three plant-derived antimicrobials (PDAs), namely carvacrol (CR), thymol (TY), and β -resorcylic acid (BR; 2,4-dihydroxybenzoic acid), were effective in reducing *Salmonella* spp. and *L. monocytogenes* in soil. Carvacrol and thymol are extracted from the essential oil of oregano (*Origanum vulgare* (Lamiaceae)) while BR is a phytophenolic compound widely distributed among angiosperms (Bolton et al., 1986). In addition, these compounds are generally recognized as safe (GRAS) by the Food and Drug Administration (FDA) (FDA, 2015; Friedman, 2003). In order to potentially use CR, TY and BR as soil amendments, their effects on the soil pH and nutrients as well as the soil microbiome must be understood since these aspects are key to plant health. Therefore, the objective of the present study was to determine the effect of CR, TY, and BR on soil pH, nutrients, and the microbiome.

2. Materials and Methods

2.1 Soil pH and nutrient analysis

Representative samples of sandy loam soil was collected from a commercial produce farm by mixing equal portions of soil from two distinct locations of the field which had not received any pesticide treatments. The soil was combined with 0.5% (w/w) of CR (Sigma-Aldrich Corp, St. Louis, MO), TY (Sigma-Aldrich), and BR (Sigma-Aldrich). A negative control was included that did not contain PDAs. The soil samples were thoroughly mixed for 2 min by hand, and stored at room temperature (23°C) which represents the soil temperature in the northeastern US during the growing season (NRCS, 2015). On days 0, 7, 14, and 21, the samples were brought to the University of Connecticut Soil Nutrient Analysis Laboratory, and tested for pH, textural class estimate, organic matter estimate, and an estimate of calcium, magnesium, phosphorus, potassium, boron, copper, iron, manganese, zinc, and aluminum concentration using the Modified Morgan extraction method (Helmke and Sparks, 1996).

The soil nutrient analysis was set up as a completely randomized design with a two-way factorial. The factors included PDA concentration and time. Treatments were done in triplicate and the experiment was repeated twice. The results were analyzed using the PROC-GLM procedure of SAS version 9.4 and significance was detected as $P < 0.05$.

2.2. Bacterial strains and sample preparation

This experiment consisted of 6 total treatments; to prepare the inoculum, four serovars of *Salmonella enterica*, including *S. Braenderup*, *S. Baildon*, *S. Newport*, and *S. Montevideo*, which had originally been isolated from tomatoes, were cultured separately in 10 ml of tryptic soy broth (TSB; Difco, Becton Dickinson, Sparks, MD) at 37°C for 24 hours. Equal volumes of the cultures were combined and sedimented by centrifuged at 3600 rpm for 15 minutes at 4°C. The pellet was resuspended in 10 ml of phosphate buffered saline (PBS; pH 7.0) and used as the inoculum (8 Log CFU/ml). A volume of 250 µl of the culture (8 Log CFU/ml) was added to 25 g of sandy loam soil in a sterile stomacher bag (Nasco, Fort Atkins, Wisconsin). The samples were then mixed by hand for 2 min, followed by the addition 0.5% CR or TY. The samples were again mixed by hand for 2 min. The various treatment groups included a negative control (no *Salmonella*, no PDA), 0.5% CR, 0.5% TY, a *Salmonella* control (*Salmonella*, no PDA), *Salmonella* + 0.5% CR, and *Salmonella* + 0.5% TY. The samples were stored at room temperature (23°C) and on days 0, 7, 21, and 42, 15 g soil aliquots from each treatment groups were removed and stored at -80°C for total microbial DNA extraction.

2.3 DNA extraction and 16Sr RNA sequencing

From each treatment groups, 0.5 g of soil sample was used for DNA extraction using the PowerSoil® DNA Isolation Kit (MO BIO, Carlsbad, CA) following the manufacturer's protocol

(MO BIO Laboratories, 2013). Total microbial DNA extracted was quantified using the Quant-iT PicoGreen kit (Invitrogen, ThermoFisher Scientific, Waltham, MA). The DNA samples were stored at -20°C and analyzed at the UConn Microbial Analysis, Resources and Services (MARS) facility for microbiome analysis. MiSeq Illumina (Illumina, Inc., San Diego, CA) PCR was used to amplify the 16S rRNA gene as per a published protocol (Kozich et al., 2013). Thirty ng of extracted DNA was used as the template for PCR, and the V4 region was amplified using 515F and 806R with Illumina adapters and golay indices at the 3' end (Caporaso et al., 2012). The samples were amplified in triplicate using Phusion High-Fidelity PCR master mix (New England BioLabs, Ipswich, MA) with the addition of 10 µg BSA (New England BioLabs). The PCR reaction was incubated at 95°C for 3.5 min, the 30 cycles of 30 s at 95.0°C, 30 s at 50.0°C and 90 s at 72.0°C, followed by a final extension as 72.0°C for 10 minutes. The PCR products were then pooled for quantification and visualization using the QIAxcel DNA Fast Analysis (Qiagen, Venlo, Netherlands). The PCR products were normalized based on the concentration of DNA from 250-400 bp, and pooled using the QIAgility liquid handling robot. The pooled PCR products were cleaned using the Gene Read Size Selection kit (Qiagen) according to the manufacturer's protocol. The cleaned pool was sequenced on the MiSeq using v2 2x250 base pair kit (Illumina, Inc).

2.4. Sequence analysis

The microbiome analysis was set up as a completely randomized design with a two-way factorial. There were four replicates per treatment per day ($n = 4$) with a total of 96 samples ($N = 96$). Sequences were filtered and clustered using Mothur 1.36.1 based on a published protocol with slight modifications (Kozich et al., 2013). The operational taxonomic units (OTUs) were clustered at 97% sequence similarity. Downstream analysis of samples was done using R version 3.2. The alpha diversity was calculated by using inverse Simpson to measure the richness and

evenness of the OTUs. The effect of both treatment and day on the alpha diversity was analyzed using Tukey's test. The beta-diversity was estimated as the difference in bacterial composition based on treatment and time by coupling Bray-Curtis Dissimilarity with principal coordinate analysis (PCoA). A permutational multivariate analysis (PERMANOVA, adonis function, 75 permutations) was conducted to analyze the effect of each treatment, and day on the bacterial community composition. Finally, the relative abundance of OTUs of major phyla, order, and genera were determined to further assess the effect of treatment. Tukey's test was used to identify changes in groups of bacteria based on treatment and the significance was detected at $P < 0.05$.

3. Results

3.1. The effect of PDAs on soil pH and nutrients

The effect of 0.5% CR, TY, and BR on the soil pH and nutrients on days 0, 7, 14, and 21 is depicted in Table 1 along with the optimal ranges for calcium, magnesium, phosphorous, and potassium and normal levels for each of the micronutrients. Carvacrol (0.5%) decreased the level of available copper on days 0 and 14, and that of zinc on day 0 ($P < 0.05$). Similarly, CR treatment reduced the available iron and aluminum levels on days 0, 7, and 14, while available manganese was increased on all tested days ($P < 0.05$). Thymol (0.5%) decreased available iron and aluminum on days 7 and 14, while the concentration of available manganese was increased on all days tested ($P < 0.05$). β -resorcylic acid (0.5%), being an organic acid, resulted in a decrease in soil pH on all days tested ($P < 0.05$) although the pH did increase over time, from an average of 4.7 on day 0 to 5.9 on day 21. Available phosphorous levels were higher on day 0 ($P < 0.05$) whereas iron, copper, manganese, and aluminum were increased on all days in BR-treated samples. Also, BR increased available magnesium levels on days 14 and 21 ($P < 0.05$). Despite the changes resulting from the addition of CR and TY, the nutrient contents remained within the normal limits for northeastern

US soils (UConn Soil Nutrient Analysis Laboratory, 2014). Notably, CR and TY enhanced the available manganese content of the soil, as they were considered low in the controls ($P < 0.05$). Similarly, BR increased available manganese levels while phosphorous and copper remained within normal limits ($P < 0.05$). Although BR increased available magnesium, the levels of this nutrient were above optimum levels in the control samples as well. The decreased pH caused by BR could be slightly too acidic for fruit and vegetable crops and the high available aluminum concentration could possibly result in toxicity to sensitive plants (UMass Amherst, 2015).

3.2. The effect of PDAs on the soil microbiome

The results on the alpha diversity based on treatment and day are shown in Figure 1a and 1b, respectively. As seen in Figure 1a, there was no difference in the inverse Simpson index of the groups CR, TY, *Salmonella* control, *Salmonella* + CR, and *Salmonella* + TY when compared with the negative control ($P > 0.05$). Although the mean diversity was slightly higher in the negative control there was also a high amount of variability and so the difference was not significant ($P > 0.05$). The inverse Simpson values for bacterial diversity were lower on day 21 when compared to days 0, 7, and 42, as depicted in Figure 1b ($P < 0.05$). On day 42, the inverse Simpson value was lower than that on days 0 and 7, but greater than on day 21 ($P < 0.05$). The results on the beta diversity are represented in Figure 2. On day 0, all the treatments were clustered, but there was a shift in the communities due to the addition of CR, TY, and *Salmonella* on each of the consecutive sampling days, with some overlap. This is apparent in Figure 2, wherein for days 7, 21, and 42, the communities were separated between two different clusters, one with the negative control and *Salmonella* control and the second cluster with phytochemical treatments. Also, within these clusters, there was a slight distinction between samples containing *Salmonella* and the ones without the pathogen. Although there were interactions between the factors ($P <$

0.05), the PERMANOVA supported the cluster analysis that the treatments (CR and TY), the presence of *Salmonella*, and day affected the community composition ($P < 0.05$). The addition of CR and TY affected the community composition the most, contributing to 18% of the composition, followed by *Salmonella* and day, each at approximately 4%.

Figure 3a depicts the relative abundance of the major phyla in the soil for each sampling day, while Figure 3b shows the phyla distribution for all days. Figure 3c depicts the relative abundance of major genera and order for each day and Figure 3d shows the genera and order with all days combined. As shown in Figure 3b, compared to the control, CR resulted in an increase in the major phyla such as *Firmicutes* and a decrease in *Chloroflexi* and *Bacteroidetes* ($P < 0.05$), whereas TY resulted in an increase of *Firmicutes* and a decrease in *Chloroflexi* group ($P < 0.05$). The addition of *Salmonella* alone resulted in an increase in *Acidobacteria*, and *Alphaproteobacteria*, and a decrease in *Actinobacteria*, ($P < 0.05$). Similarly, *Salmonella* + CR had the same effect as CR alone along with a decrease in *Betaproteobacteria* ($P < 0.05$). In addition, *Salmonella* + TY resulted in a decrease in *Chloroflexi*, *Bacteroidetes*, and *Betaproteobacteria* ($P < 0.05$). As observed in Figure 3d, when compared to the controls, CR and TY increased *Bacillus* populations significantly ($P < 0.05$). *Salmonella* + CR also increased *Bacillus* compared to the negative control and the *Salmonella* control ($P < 0.05$). Although *Salmonella* + TY did not increase *Bacillus* community as compared to the negative control ($P > 0.05$), the *Bacillus* group was increased with respect to *Salmonella* control ($P < 0.05$). Additionally, the treatment groups *Salmonella* + CR and *Salmonella* + TY contained a lesser proportion of *Burkholderiales* ($P < 0.05$). When compared to the controls, *Rhizobiales* appeared to be slightly increased by CR and TY, but the difference was not significant ($P > 0.05$). However,

the addition of CR and TY in the *Salmonella* + CR and *Salmonella* + TY groups, respectively, increased *Rhizobiales* compared to the *Salmonella* control.

4. Discussion

The rate of produce-associated outbreaks has been increasing in recent decades and agricultural soil is a potential route of transmission for pre-harvest contamination with foodborne pathogens (Doyle & Erickson, 2008; Greene et al., 2008). Therefore, a natural and environment friendly soil amendment could be beneficial in reducing pathogens in soil. The PDAs, CR, TY, and BR have been shown to be effective in reducing a number of foodborne pathogens on produce (Baskaran et al., 2013; Burt, 2004; Mattson et al., 2011) and evidence suggests that these phytochemicals selectively kill pathogens without adversely affecting commensal bacteria (Hawrelak et al., 2009; Thapa et al., 2012; Upadhyaya, 2015a,b). This effect is an important attribute for soil amendments, as the diversity of soil bacteria is important for maintaining plant health (Raaijmakers et al., 2009; Zuberer, n.d.). This study demonstrated that CR and TY could potentially be used as a soil amendment because of their minimal effects on soil nutrients and microbial diversity.

For the Modified Morgan extraction method, the optimum conditions for pH, calcium, magnesium, phosphorous, and potassium were considered as 6 and 6.8, 2,016 and 2,687 kg/ha, 196 to 279 kg/ha, 16 and 22 kg/ha, and 280 to 391 kg/ha, respectively (UConn Soil Nutrient Analysis Laboratory, 2004). Normal ranges of trace elements in the northeastern US are between 0.1 and 2.0 ppm for boron, 0.3 and 8.0 ppm for copper, 1.0 and 40.0 ppm for iron, 3.0 and 20.0 ppm for manganese, 0.1 to 70.0 ppm of zinc, and between 10 to 300 ppm of aluminum (UConn Soil Nutrient Analysis Laboratory, 2014). Aluminum is not considered an essential nutrient, but

if the pH is below 5.5 the amount of available aluminum may reach a level high enough to cause toxicity to sensitive plants such as lettuce, carrots, and beets (UMass Amherst, 2015).

The results for the soil nutrient analysis revealed that CR resulted in a change in available copper, iron, manganese, and zinc content of soil on at least one of the sampling days. Similarly, TY resulted in a change to available iron, manganese, and aluminum on at least one of the days. However, none of these changes seem likely to be detrimental to plant health because all of the values remained within normal limits for northeastern US soils. Manganese was slightly below normal levels in the control and the addition of CR and TY increased it to more acceptable levels. Addition of BR decreased the pH to slightly below optimal levels and although the available aluminum remained within normal levels, it could potentially cause toxicity to sensitive plants (UMass Amherst, 2015). Additionally, all samples had acceptable medium-level organic matter content. Nitrogen content was not analyzed in this test because it is highly variable and the recommended amount to be added to fields depends on many different environmental factors (UConn Soil Nutrient Analysis Laboratory, 2014).

The diversity of the soil microbiome is very important to maintain plant health. The genera *Pseudomonadales*, *Burkholderia*, and *Bacillus* are known to be beneficial to plant health as well as the nitrogen-fixing bacteria of the order *Rhizobiales* (Osorio Vega, 2007; Raaijmakers et al., 2009). In this study, the effect of CR and TY addition, as well as *Salmonella* on the soil microbiome was analyzed over time. *Salmonella* was added to determine the effect it exerts on the soil microbial composition, and how the addition of PDAs may alter the same. None of the treatments significantly affected the alpha diversity of the soil microbiome as expressed in terms of inverse Simpson index ($P > 0.05$) and hence the soil retained a high amount of microbial

diversity in the presence of CR and TY.. However, on days 21 and 42, lower inverse Simpson values were observed when compared to days 0 and 7.

To compare the community differences between treatments, beta diversity was analyzed using Bray-Curtis dissimilarity. Although there was some overlap between the bacterial communities, factors including treatment and day caused a shift in the community structures ($P < 0.05$). To determine which groups of bacteria were affected by each of the treatments, the ratio of major phyla, order, and genera were further analyzed. Although there were minor differences in the phyla such as *Firmicutes*, *Chloroflexi*, *Bacteroidetes*, *Acidobacteria*, *Actinobacteria*, *Alphaproteobacter*, and *Betaproteobacter*, none of the changes are considered detrimental. Notably, CR and TY increased the PGPB *Bacillus* and TY increased *Pseudomonadales*; however this change was not significant, most likely due to variability. Furthermore, CR and TY increased the nitrogen fixing bacteria *Rhizobiales* as compared to the *Salmonella* control, supporting that the plant compounds may help restore contaminated soil to a healthy microbial condition.

There is evidence that indicates that many plant compounds are biodegradable and would not persist in the environment for extended periods of time to exert any negative effects (Heider and Fuchs, 1997; Isman, 2006). Additionally, at present there are no known reports of pathogens developing resistance to the plant compounds due to their multiple antimicrobial mechanisms (Ohno et al., 2003; Domadia et al., 2007). Overall, CR and TY have the potential as environmental friendly soil amendments as they do not detrimentally alter the soil pH, nutrient levels, and microbiome, and can potentially restore contaminated soil to a healthier microbial composition. However, further studies under field conditions are warranted to determine their environmental degradation and effect on plant growth.

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Table 1.

The effect of 0.5% CR and TY on the soil pH and nutrients. The error values represent SEM (n=6). Sandy loam soil was combined with CR or TY at 0.5%. The samples were homogenized and stored at room temperature. On days 0, 7, 14, and 21 the samples were subjected to nutrient analysis (Modified Morgan).

1.

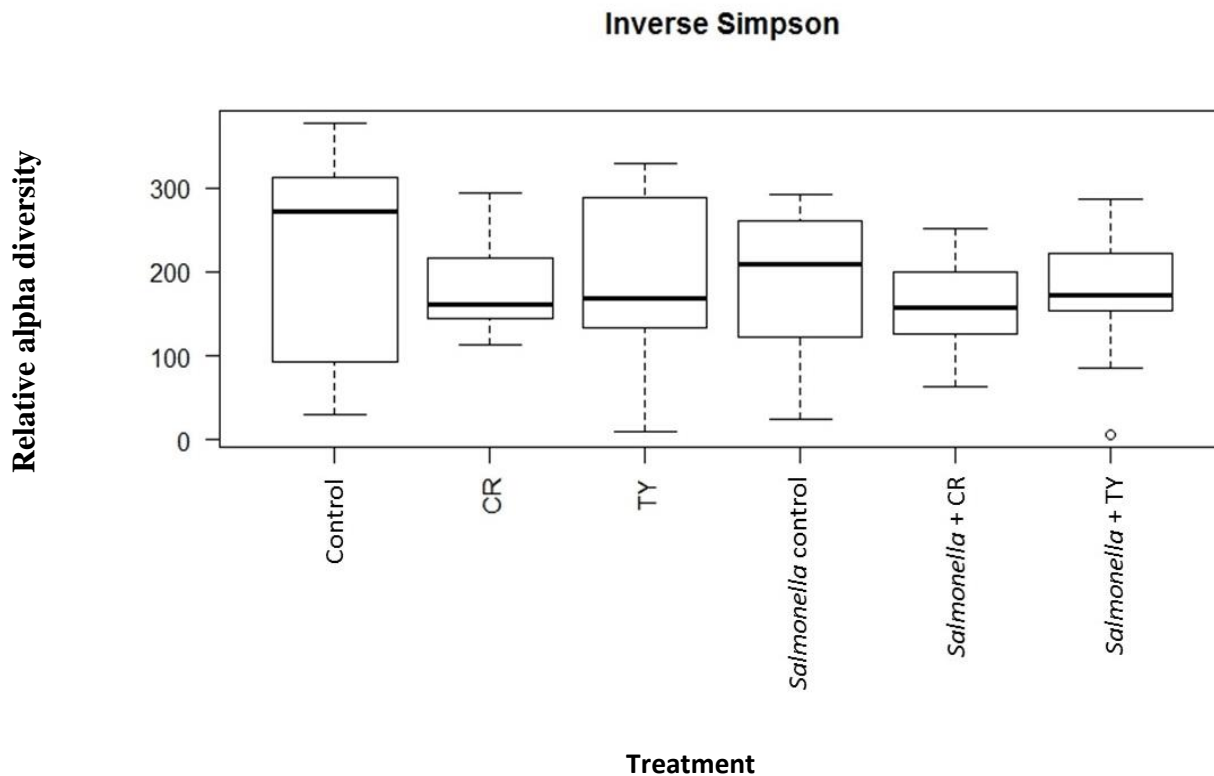
	pH	calcium (lbs/acre)	magnesium (lbs/acre)	phosphorous (lbs/acre)	potassium (lbs/acre)	boron (ppm)	copper (ppm)	iron (ppm)	manganese (ppm)	zinc (ppm)	aluminum (ppm)
Optimal/Normal Range	6 - 6.8	2,016 - 2,687	196 - 279	16 - 22	280 - 391	0.1 - 2.0	0.3 - 8.0	1.0 - 40.0	3.0 - 20.0	0.1 - 70.0	10 - 300
Day 0											
Control	6.6±0.01	2195±47.83	496±1.70	59±0.34	229±5.86	0.45±0.00	2.00±0.01	9.85±0.10	1.95±0.01	6.57±0.21	58±0.48
0.5%CR	6.6±0.01	2599±12.69	466±4.19	53±0.52	255±1.67	0.37±0.01	1.85±0.00 *	7.05±0.08 *	3.87±0.02 *	4.10±0.02 *	50±0.22 *
0.5% TY	6.6±0.01	2690±24.02	474±5.74	56±0.63	263±2.14	0.38±0.01	1.93±0.01	7.78±0.18	4.23±0.04 *	4.78±0.04	52±0.54
0.5% BR	4.7±0.00 *	2577±14.25	465±5.03	77±0.91 *	277±2.86	0.43±0.01	2.57±0.01 *	38.73±0.24 *	8.47±0.05 *	5.57±0.07	155±0.78 *
Day 7											
Control	6.6±0.01	2514±9.93	455±3.42	49±1.94	229±2.44	0.40±0.01	1.95±0.00	9.12±0.07	1.60±0.01	4.45±0.08	58±0.32
0.5%CR	6.6±0.01	2595±9.81	463±3.97	56±0.48	247±1.87	0.38±0.01	1.90±0.01	6.77±0.05 *	4.90±0.07 *	4.03±0.05	52±0.22 *
0.5% TY	6.6±0.01	2539±13.97	463±5.71	56±0.56	250±1.38	0.38±0	1.92±0.01	7.45±0.07 *	4.18±0.04 *	4.02±0.03	53±0.23 *
0.5% BR	5.6±0.02 *	2522±10.29	485±4.34	65±0.27	266±2.96	0.40±0.01	2.65±0.01 *	44.45±0.64 *	12.72±0.12 *	5.12±0.03	156±1.36 *
Day 14											
Control	6.6±0.02	2728±54.95	446±2.57	57±0.73	296±2.38	0.42±0.01	2.08±0.01	8.28±0.02	2.03±0.08	5.25±0.09	57±0.58
0.5%CR	6.6±0.01	2594±13.68	466±1.55	60±0.70	303±1.35	0.43±0.01	1.93±0.00 *	6.53±0.05 *	4.67±0.02 *	5.00±0.08	50±0.29 *
0.5% TY	6.6±0.01	2577±20.79	456±2.05	59±0.90	287±0.97	0.38±0.01	2.00±0.01	6.25±0.02 *	4.35±0.06 *	4.87±0.11	49±0.32 *
0.5% BR	5.8±0.04 *	2663±17.10	497±1.98 *	63±0.61	295±1.57	0.38±0.01	2.58±0.01 *	34.87±0.96 *	12.88±0.05 *	6.67±0.15	135±2.65 *
Day 21											
Control	6.6±0.02	2485±16.50	432±1.84	57±0.34	282±0.68	0.40±0.01	1.92±0.03	8.23±0.22	2.70±0.08	4.50±0.05	55±1.23
0.5%CR	6.6±0.02	2580±18.86	449±3.08	63±0.72	300±2.29	0.42±0.01	1.80±0.02	6.62±0.19	4.45±0.02 *	3.95±0.05	50±1.31
0.5% TY	6.6±0.01	2465±13.66	446±1.82	62±0.67	295±2.41	0.40±0.01	1.83±0.03	7.07±0.26	4.45±0.02 *	4.57±0.10	51±1.41
0.5% BR	5.9±0.01 *	2607±20.43	494±1.66 *	66±0.74	298±1.66	0.40±0.01	2.47±0.03 *	29.87±0.39 *	12.78±0.03 *	6.70±0.26	119±1.61 *

* Denotes treatment value is significantly different when compared to control within the day

Fig. 1.

The alpha diversity represented as the inverse Simpson ratio. Sandy loam soil was either with or without ~ 6.0 Log CFU/g was mixed with CR and TY at a concentration of 0.5%. On days 0, 7, 21, and 42 DNA was isolated from the soil using the MO Bio PowerSoil® DNA Isolation Kit and sent to the UConn MARS facility for analysis. MiSeq Illumina PCR was used to amplify the 16S rRNA gene (V4), the data was clustered using Mothur version 1.36.1, and downstream analysis was done using R version 3.2. **(a)** The inverse Simpson ratio for each treatment. **(b)** The inverse Simpson ratio for each day.

a.



b.

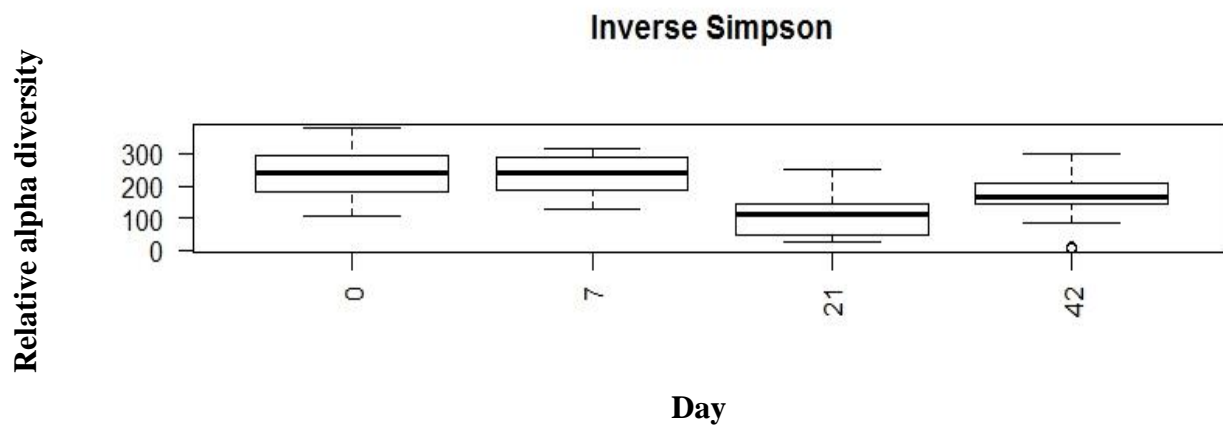


Figure 2.

Bray-Curtis beta diversity. Sandy loam soil was either with or without ~ 6.0 Log CFU/g was mixed with CR and TY at a concentration of 0.5%. On days 0, 7, 21, and 42 DNA was isolated from the soil using the MO Bio PowerSoil® DNA Isolation Kit and sent to the UConn MARS facility for analysis. MiSeq Illumina PCR was used to amplify the 16S rRNA gene (V4), the data was clustered using Mothur version 1.36.1, and downstream analysis was done using R version 3.2.

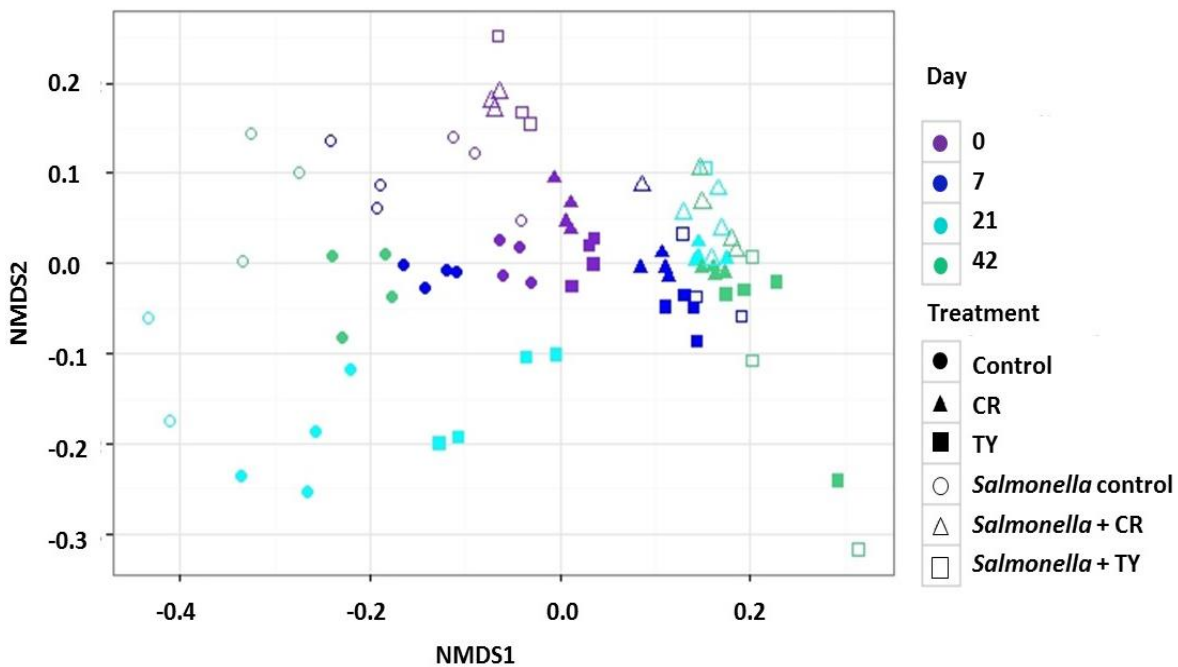
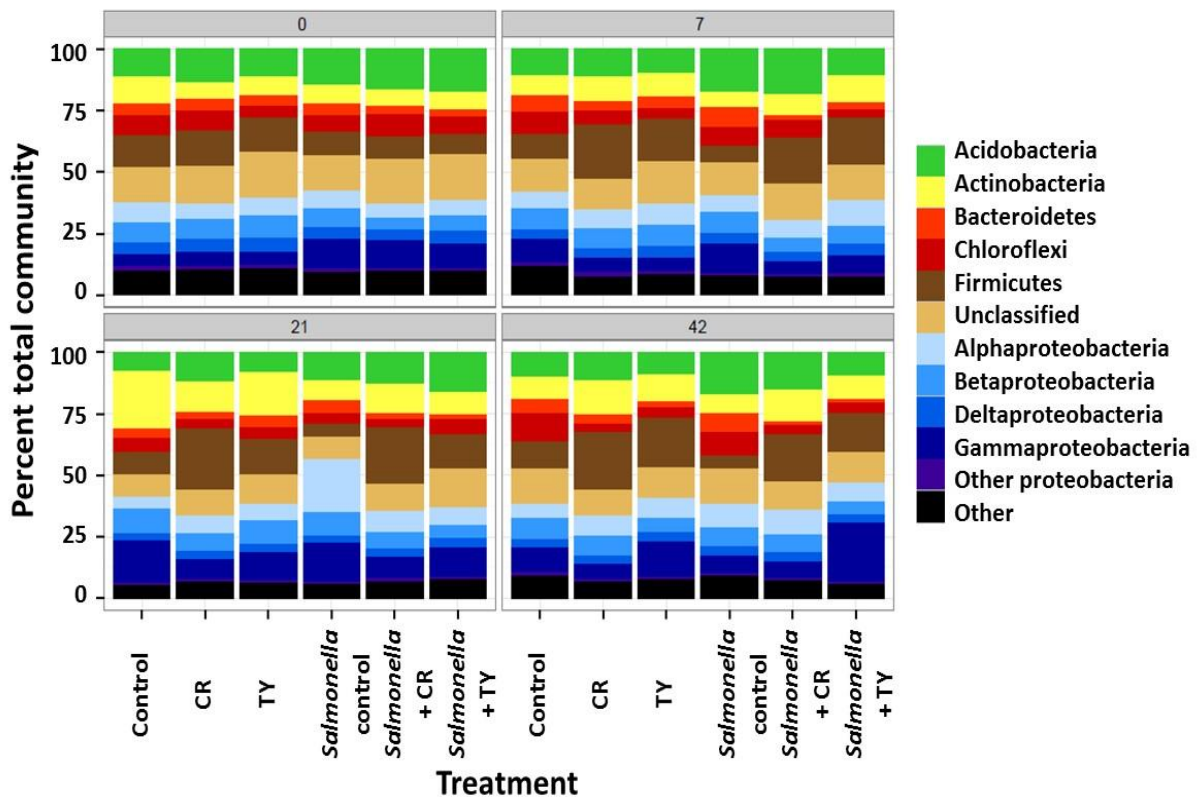


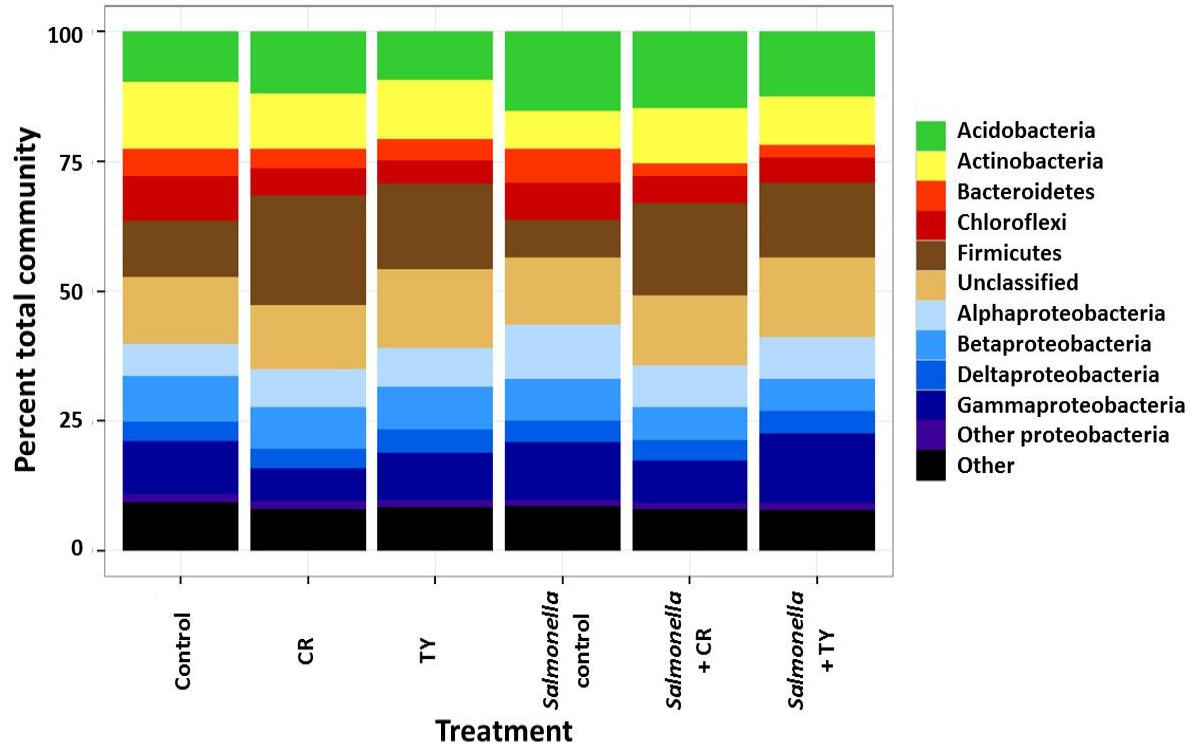
Figure 3.

Changes in major phyla, order, and genera. Sandy loam soil was either with or without ~ 6.0 Log CFU/g was mixed with CR and TY at a concentration of 0.5%. On days 0, 7, 21, and 42 DNA was isolated from the soil using the MO Bio PowerSoil® DNA Isolation Kit and sent to the UConn MARS facility for analysis. MiSeq Illumina PCR was used to amplify the 16S rRNA gene (V4), the data was clustered using Mothur version 1.36.1, and downstream analysis was done using R version 3.2. **(a)** Ratio of major phyla as shown for each day. **(b)** Ratio of major phyla with days combined. **(c)** Ratio of PGPB as shown for each day. **(d)** Ratio of PGPB with days combined.

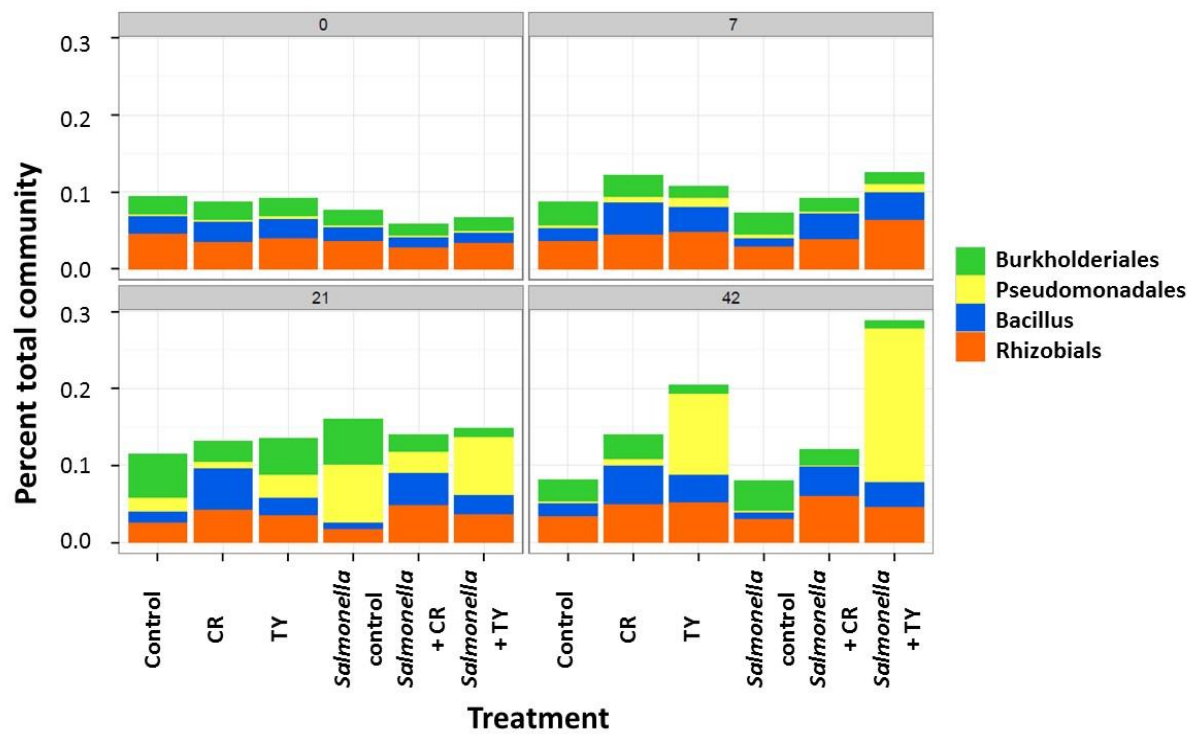
a.



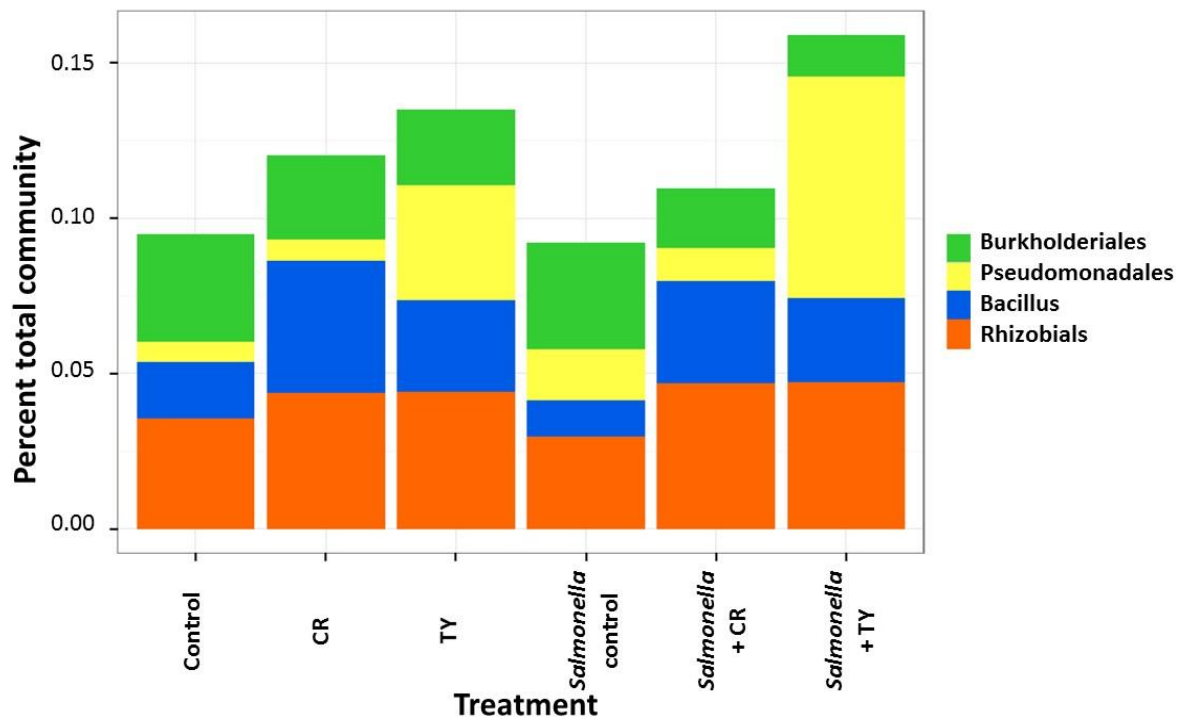
b.



c.



d.



Chapter V

Summary

Salmonella and *Listeria monocytogenes* are two major pathogens associated with foodborne disease outbreaks linked to produce. These pathogens can cause pre-harvest contamination of produce because of their persistence in agricultural soil, thereby highlighting the need for strategies for reducing microbial pathogens in soil. However, limited studies have been done on the use of a soil amendment to reduce foodborne pathogens in the field.

This study determined the potential for three phytochemicals, namely carvacrol (CR), thymol (TY), and β -resorcylic acid (BR; dihydroxybenzoic acid) as soil amendments to reduce *Salmonella* and *L. monocytogenes*. The first objective of this study determined the efficacy of CR, TY, and BR in reducing *Salmonella* and *L. monocytogenes* in agricultural soil during a storage period of three weeks. It was found that 0.25 and 0.5% CR and 0.5% TY were able to reduce both *Salmonella* and *L. monocytogenes* to undetectable levels by day 1 in sandy loam soil. By day 21 all of the compounds decreased *Salmonella* and *L. monocytogenes* to ~ 1 Log CFU/g.

The second objective of this study investigated the effect of phytochemicals on soil pH, nutrients, and microbiome. The addition of CR and TY resulted in minor changes to some of the nutrients, but all remained within acceptable limits. However, β -resorcylic reduced the soil pH significantly, and therefore increased the amount of available aluminum. Carvacrol and TY also did not detrimentally affect the soil microbiome significantly. The soil retained a high level of diversity despite minor changes in some phyla. Carvacrol and TY also increased the plant growth-promoting bacteria and restored the population of the nitrogen-fixing bacteria *Rhizobiales*, which had decreased in the presence of *Salmonella*.

The results of this study suggest the potential use of CR, TY, and BR as soil amendments to kill *Salmonella* and *L. monocytogenes* for improving the microbiological safety of fresh

produce. However, long-term field trials should be conducted to determine environmental fate of these phytochemicals and their effect on plant growth.