ABSTRACT

Title of Thesis:	The Plant Rhizosphere-Phyllosphere Connection: Analysis of root-associated *Pseudomonas* sp. -induced changes in Fruit Surface Phytochemical Profiles of Heirloom and Modern Tomato cultivars and effect on *Salmonella enterica* association
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Despite more rigorous food safety regulation for fruit and vegetable production, foodborne illnesses and massive food recalls have resulted from the enteropathogen *Salmonella enterica* on *Solanum lycopersicum* (tomato) fruits. While some phytochemical profiles have been studied on modern cultivars in regards to minimizing *Salmonella*-plant association, little research has been done on heirloom varieties. The interaction between the plant rhizosphere and phyllosphere remains understudied and the inoculation of plants with plant-growth promoting rhizobacteria (PGPR) can modify phyllosphere components of plants including fruit properties. No research exists on whether PGPR colonization of tomato plant roots can alter the fruit's phytochemical profile to affect *Salmonella* association. Through several chemical and microbial analyses, I investigated the *Salmonella* association with fruit of modern and heirloom cultivars, as well as the impact of PGPR inoculation on this system. In Chapter 3, I assessed fruit of modern and heirloom varieties, and explored possible associations between *Salmonella* growth in fruit soil amendments to a tomato field and profiling of

surface washes and total sugar quantifications, citric acid and (for heirloom varieties) the fatty acid profiles. Microbial counts differed from variety to variety and heirloom varieties demonstrated higher levels of sugars and citric acid compared to modern varieties. Total sugar quantifications and citric acid were correlated with microbial counts in heirloom varieties, but not in modern varieties. Chapter 4 focuses on the effects of inoculating the heirloom varieties with a PGPR in the genus *Pseudomonas* sp.on the fruit surface phytochemical profile and changes in microbial association. Differing trends between the PGPR inoculated and non-inoculated groups were seen where citric acids were correlated with *Salmonella* association for the non-PGPR group, but not for the PGPR inoculated group. For both the PGPR inoculated and non-inoculated groups, we measured a high degree of variation between the cultivars for the phytochemical profiles, and for both groups hexadecanoic acid was negatively correlated with microbial counts. This study furthers the understanding of the relationship between tomato fruit phytochemistry and *Salmonella* association, as well as the effects of PGPR on modifying tomato fruit phytochemistry. These findings can help manage and improve the pre- and post-harvest food safety of tomato fruit.

The Plant Rhizosphere-Phyllosphere Connection: Analysis of root-associated *Pseudomonas* sp. -induced changes in Fruit Surface Phytochemical Profiles of Heirloom and Modern Tomato cultivars and effect on *Salmonella enterica* association

by

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Chapter 1: Introduction

The enteric pathogen *Salmonella* has been known to cause humans illness known as salmonellosis. Despite good food safety practices, *Salmonella* presents immense public health concern (CDC, 2019) and financial concern to both farmers and consumers (Ribera et al, 2012). In recent years there have been a number of *Salmonella* outbreaks implicating tomato (*Solanum lycopersicum*) in various parts of the world (CDC, 2005), (CDC, 2007), (Greene et al; 2007).

Finding effective ways to minimize *Salmonella enterica* subsp. *enterica* association on tomato (*Solanum lycopersicum*) fruit surfaces can reduce the chance of foodborne illness. The *Salmonella*-tomato association is tomato cultivar-dependent (Han and Micallef, 2014). One of the drivers of this variation in bacterial association appears to be related to differential levels of phytocompounds among fruit of different cultivars that can impact bacterial-fruit associations. Sugars, sugar alcohols and organic acids in tomato fruit washes were associated with increased *Salmonella* growth in these solutions, while medium- to long-chain saturated fatty acids and the unsaturated oleic acid were negatively correlated (Han and Micallef, 2016). Further investigation into the effect of saturated medium-chain fatty acids on *Salmonella* growth confirmed the inhibitory effect of these compounds (Dev Kumar and Micallef, 2017).

A potential way to modify the specialized metabolites on the surface of tomato fruit could be through the root inoculation of plant-growth promoting-rhizobacteria (PGPR). One plant-growth-promoting rhizobacterial species of interest is *Pseudomonas* sp. S4. This PGPR strain has been shown to increase plant vigor and biomass accumulation in tomato plants as well as reduce the association of *Salmonella* with leaf surfaces (Hsu and Micallef, 2017). The mechanism by which root colonization with *Pseudomonas* sp. S4.is able to restrict this plant-bacterial

association is not known. Moreover, the metabolic influences of these PGPR interactions on the plant, and specifically on the fruit surface remain a mystery. However, there has been research regarding similar plant-growth-promoting-rhizobacteria modifying fatty acid levels in plants. Taken together, these findings could suggest that inoculation of tomato plants with *Pseudomonas* sp. S4 may very well increase specific fatty acid content in the fruit surfaces.

I, therefore, hypothesize that tomato fruit surfaces with higher medium- to long-chain saturated fatty acids and unsaturated oleic acid will demonstrate a lesser association with *S. enterica*. The second hypothesis is that tomato plants inoculated with *Pseudomonas* sp. S4 will demonstrate higher levels of medium- to long -chain saturated fatty acids and unsaturated oleic acids, in turn modulating the *Salmonella*-tomato association.

Objective: The overall goal of this research is to discover and understand strategies to minimize *S. enterica* association with *S. lycopersicum* fruit. In the specific objectives of this project, I set out to screen modern and heirloom *S. lycopersicum* fruit surfaces for fatty acids and to understand the role of *Pseudomonas* sp. S4 in the potential modifications of metabolites of the fruit surfaces.

Hypothesis 1, Sugars and Citric Acid analysis: Sugars and citric acid will be associated with increased *Salmonella* growth for both modern and heirloom varieties

Hypothesis 2, Fatty Acid Analysis: Fatty acids will be associated with inhibited *Salmonella* association

Hypothesis 3, PGPR Analysis: Application of PGPR will be associated with higher fatty acids and inhibited *Salmonella* association.

Specific Aims:

- Aim 1: Screen and analyze modern and heirloom tomato cultivar fruit surfaces for degree of *Salmonella* association
- Aim 2: Screen and analyze tomato cultivars for total sugars and citric acid
- Aim 3: Screen and analyze tomato cultivars for fatty acid types and levels
- Aim 4: Compare *Salmonella* association, fatty acids, citric acid, and total sugars between PGPR +/- groups
- Aim 5: Investigate the impact of PGPR on fruit color and texture traits and relation to *Salmonella* association

Chapter 2: Literature Review

2.1. Foodborne illnesses due to fresh produce

The enteric pathogen *Salmonella* has been known to cause humans illness known as salmonellosis. According to the Center for Disease Control and Prevention (CDC), consumers can become infected from a number of agricultural products including animal products and fresh produce. The illness is particularly dangerous in children, the elderly, and those with immunocompromised health conditions and results in an estimated 1.35 million illnesses, 26,500 hospitalizations, and 420 deaths in the United States every year (CDC, 2019) (CDC 2020). Furthermore, the financial impacts of foodborne illness outbreaks and deaths in the US are estimated to be between $5-6 billion a year (Ribera et al, 2012). Additionally, the CDC linked 46% of foodborne illness to fresh fruits and vegetables (Painter et al; 2013). In recent years there have been a number of *Salmonella* outbreaks implicating tomato (*Solanum lycopersicum*) in various parts of the world (CDC, 2005), (CDC, 2007), (Greene et al; 2007). There are a number of contamination sources for *Salmonella* including manure composts and irrigation water that contribute to cross-contamination of tomato plants (Jablasone et al, 2004, Islam et al, 2004). The persistence of *Salmonella* in soil environments is dependent on a number of conditions including microbial diversity, where the higher level of diversity in indigenous microflora, the lower the rate of *Salmonella* survivability, while the lower level of indigenous microflora diversity, the higher the rate of *Salmonella* survivability (Zibilske and Weaver 1978). Persistence of *Salmonella* in soil conditions is also dependent on abiotic conditions including air temperature and moisture where cooler and wetter conditions were favorable to survivability in the soil (Lee et al, 2019).

2.2. *Salmonella* in the Plant Phyllosphere

Epiphytic attachment and colonization of *Salmonella* on plants has been explored on a number of different plants for both seeds and leaves. Damaged seeds and rougher, grooved surfaces demonstrated higher rates of association with *Salmonella* as opposed to undamaged seeds and smoother surface seeds. Additionally, the use of fungicides on seed surfaces generally did not contribute to a reduction of microbial association (Cue and Chen, 2017). Differences in the morphological structure in lettuce leaves also resulted in different levels of attachment between the plant host and *Salmonella* where the base of leaves harbored microbial growth more than middle and upper portions of the leaves. However, neither leaf grooves nor stomatal density could explain the differences in microbial association. (Van der Linden et al, 2016).

Interestingly in other studies, internalization of tomato plant tissues was linked to chemotaxis and light induced opening of stomata (Kroupitski et al, 2009). In the epiphytic regions of plants, *Salmonella* attachment is dependent upon a number of microbial advantages including flagella for motility and curli fimbriae for adhesion to surfaces. Cellulose was seen as a major component of plant tissue that aided in microbial association. *Salmonella* also utilizes exopolymeric substances in biofilm formation to maintain communal growth in the face of biotic and abiotic stresses (Maruzani et al, 2018; Yaron et al, 2014). Once internalized in plant tissues, *Salmonella* continues to persist and colonize tomato plants by means of *de novo* biosynthesis of amino acids, lipopolysaccharides, and nucleotides, as well as iron acquisition and maintenance of cell structure (de Moraes et al, 2017; de Moraes et al, 2018). At high levels of inoculation, *Salmonella* can move freely through the interior of tomato plants and migrate to different portions of the plant (Gu et al, 2011). It is also possible that universal stress proteins play an

important role in *Salmonella* being able to mitigate stresses in plant interiors (Kroupitski et al, 2019). A number of genes in *Salmonella* were determined to be important for internal plant colonization including stomatal opening, biosynthesis of surface appendages, and callose deposition (Montano et al, 2020). In tomato plants specifically, *Salmonella* was seen to have upregulated oxidative and nitrosative stress response genes as well as genes related to sulfur metabolism and anaerobic respiration in order to combat harsh internal plant conditions (Han et al, 2020). Enteric human pathogens like *Salmonella* have a multitude of different abilities to survive and persist not only in the environment, overcoming stressful abiotic conditions, but also colonize and persist in the plant phyllosphere and the plant interior.

2.3. Plant Defenses

Plants have a multitude of defensive mechanisms to deal with biotic stresses and pathogenic infections including both physical and chemical, induced and constitutive mechanisms. Some primary metabolites like amino acids provide broad spectrum protection against bacterial pathogens, fungi, and insects (Zaynab et al, 2019). Other constitutive chemical defenses include secondary metabolites that act as toxins that deter predators such as alkaloids and terpenoids (Wittstock and Gershenzon 2002). Physical constitutive defenses include barriers such as the waxy cuticle that not only prevent plants from water loss and abiotic stresses, but also act as a strong barrier against pests and pathogens from being able to enter the plant interior. Furthermore, some of these lipids in the surface of the waxy cuticle can act as chemical defenses against pathogens (Reina-Pinto and Yephremov, 2009). Aside from constitutive physical and chemical layers of defense on the exterior and interior of the plant, the induced pathogen/microbe-associated molecular patterns (PAMP/MAMP)-triggered immunity (PTI)

defense response is considered the first line of defense in plants. PAMP/MAMPs are highly conserved and specific molecular markers on the surface of pathogens and microbes that are recognized by host plant cell surface receptors compounds known as pattern-recognition receptors (PRRs). Many bacterial pathogens have flagella which are utilized for motility. Flagellin is well-known PAMP that host plant PRRs, specifically FLS2 and BAK1 receptor complex, can detect. The recognition of these surface receptors by the plant host in turn activates PTI, which results in a calcium signaling cascade system to induce a multitude of chemical and defense responses to combat the pathogenic microbes commonly known as hypersensitive response (HR) and induced systemic acquired response (SAR). While HR is a more localized defense response that includes callose deposition, SAR activates chemical defenses such as a reactive oxygen species burst and expression of defense genes including upregulation of secondary metabolites. Various motile plant signaling pathways are vital to SAR including salicylic acid, but the entirety of these mechanisms are still being investigated.

Pathogenic microbes have developed ways to combat PTI using effector proteins that can reduce PTI defense responses. This then activates the second line of plant immunity against pathogens known as effector-triggered immunity (ETI). This then brings about the second line of plant immunity against pathogens known as effector-triggered immunity (ETI). In ETI, plants utilize leucine-rich repeating (LRR) resistance proteins (R proteins), which are highly specific to different microbe effectors, to target pathogen effectors in the plant cell interior and bring about ETI in plant immunity to combat infection., When R proteins are not present, pathogens are able to bypass ETI and continue infection of plant hosts known as effector triggered susceptibility (ETS) (Yuan et al, 2021; Wu et al, 2014; Pruit et al, 2021; Keinath et al, 2010; Naveed et al,

2020). The realm of plant immunity is highly important to many researchers and the ability to resist and mitigate infection from pathogens, but there is less research on plant immune systems and human enteric pathogens.

2.4. *Salmonella* and plant immunity

While *Salmonella* is not considered a plant pathogen, there is mounting evidence that human pathogens on plants can be sensed by plant immune systems. Most notable, the flagellin flg22 peptide PAMP that *Salmonella* has is recognized by the FLS2 and BAK1 receptor complex across plant species. The receptor complex initiates PTI and various defenses strategies including ROS accumulation, stomatal closure, and SA accumulation. There is also a small amount of evidence that *Salmonella* not only has functional type III secretion system (T3SS) effectors coded by genetic SPI-1 and SPI-2 areas of its' genome, but that *Salmonella* may utilize T3SS effectors in order to bypass PTI and activate some symptoms of ETI. While there is more research needed on this area, there is presently some evidence suggesting that enteric human pathogens like *Salmonella* elicits at least a PTI immune response from plants, and perhaps a limited ETI response as well due to evolution in its' effector proteins (Garcia and Hirt 2014; Meng et al, 2013; Zarkhani and Schikora 2021). *Salmonella* has been shown to have regulon genes encoding to mitigate of ROS and reactive nitrogen species (RNS) damage (Han and Micallef 2020). There is some evidence that the accumulation of these reactive compounds in plants may reduce levels of *Salmonella* levels in tomato plants. Upon recognition of *Salmonella* by tomato leaves nitric oxide (NO) and ROS were upregulated and significantly limited *Salmonella* microbial populations (Ferelli et al; 2020). Other metabolites in tomato fruits have been identified as inhibitory to *Salmonella* growth including lauric and myristic acids in one

study (Kumar and Micallef 2017). Further studies in the metabolomics of tomato plants suggest that certain metabolites like sugars, sugar alcohols, and organic acids were associated with enhanced *Salmonella* growth, while various fatty acids were associated with limited *Salmonella* growth (Han and Micallef 2016). There is a complex balance between beneficial and harmful metabolites in tomato plants and the ability to modulate association between *Salmonella* and tomato plants.

2.5. *Salmonella*-plant pathogen-host plant interactions

An area of major concern is how the prevalence of phytopathogens may be able to increase the level of association between enteric pathogens like *Salmonella* and host plants. In one study involving tomato plants, *Pectobacterium carotovorum*, a soft rot phytopathogen, was hypothesized to release starches that would benefit the growth of *Salmonella*, but this was not proven accurate. Instead, a metabolic shift in *Salmonella* was detected with the co-inoculation with the phytopathogen, suggesting that *Salmonella* is able to synthesize amino acids and nucleotides more effectively as means of energy sources (George et al; 2013). In another study of the early development tomato phyllosphere, while *Salmonella* was able to reduce the levels of both phytopathogens *Clavibacter michiganensis* and *Xanthomonas gardneri*, only the presence of *C. michiganensis* managed to allow for an increase in *Salmonella* population suggesting that different phytopathogens have different impacts on the association between human pathogens and host plants (Rajashekara et al; 2020). In another study, *Salmonella*, and bacterial wilt pathogen, *Ralstonia solanacearum*, demonstrated an increase in *Salmonella* growth in various portions of tomato plants suggesting a symbiotic relationship upon co-inoculation (Pollard et al; 2014).

2.6. Plant-Growth Promoting Rhizomicrobes

Plant-growth promoting rhizomicrobes (PGPR) are beneficial microbes (bacteria or fungi) present in the rhizosphere that engage in a mutualistic and often symbiotic relationship with the root systems of a multitude of plants. Many of these microbes have been identified in their ability to solubilize nitrogen and phosphorus in the soil to be more bioavailable to plants and potentially act as biofertilizers in a hypothetical effort to limit the use of costly chemical fertilizers and pesticides and consequently limit the emission of greenhouse gasses from traditional agricultural practices (Vessey 2003). Additionally, PGPR have been known to promote physical plant growth by inducing upregulation of plant growth hormone biosynthesis such as IAA (Yousef 2018). Meanwhile in the phyllosphere, phytohormones such as ethylene are promoted as a means to improve crop production. There is also evidence that PGPR can result be sensed and identified by plant immune systems in similar ways that plant pathogens do, but plants are able to recognize PGPR from rhizosphere phytopathogens and allow their interactions with the host plant (Bhattacharyya and Jha 2012). The resulting anti-microbial defense responses from ISR in the form of increased accumulations of secondary metabolites (particularly volatile compounds) and general niche exclusion of microbe-plant symbiosis provide evidence that PGPR could also be used as effective biocontrol agents against phytopathogens (Mohammad et al; 2009, Beneduzi et al; 2012, Kannojia et al; 2019). Plant-growth promoting rhizobacteria *Ochrobactrum intermedium* was able to modify the fatty acid contents of *Arachis hypogaea* (Paulucci et al, 2015). *Rhizobium* strain TVPo8 increased fatty acids in pepper plants (Silva et al, 2014). Applications of plant growth promoting rhizobacteria including *Bradyrhizobium. japonicum, Azosprillum. lipoferum,* and *Pseusomonas.* sp. in soybeans increased unsaturated fatty acids while decreasing saturated fatty acids (Seyed 2016).

Pseudomonas. sp. was also used to increase oil content in pumpkin (Habibi et al, 2011). For tomato plants, one of the most commonly used PGPR bacterial strains is *Pseudomonas* sp. which has been shown to increase shoot dry weight, antioxidant and lycopene levels, and reduce negative impacts from phytopathogens such as *Rothia* spp. by means of proline and polyphenol production. Interestingly, more beneficial impacts of PGPR *Pseudomonas* spp. were seen in tomato plants when co-inoculated with other PGPR strains of the same genus, suggesting potential symbiosis and synergism between bacterial PGPR strains (Almaghrabi et al; 2013, Ordookhani et al; 2010, Bano and Muqarab 2017).

2.7 Plant-Growth Promoting Rhizomicrobes and Human Pathogens on Plants

The ability of strains of PGPR to reduce enteric human pathogens associating with host plants has been explored to a small degree. In one study, PGPR bacterial strain *Bacillus subtilis* was inoculated into the root systems of leafy greens lettuce and spinach. While stomatal closure was induced from the PGPR while the plants were in the presence of *Listeria* and *Salmonella* separately, the presence of only *Listeria* was diminished from the PGPR inoculation while *Salmonella* was not (Markland et al; 2015). In another study on tomato plants, *Paenibacillus alvei*, was first isolated from the phyllosphere of field tomato plants and then screened against *Salmonella* in the fruits, leaves, and blossoms of new tomato plants. Results indicated significant reduction, and in some cases full elimination of *Salmonella* microbial population from the tomato plant (Allard et al; 2014). Further studies showed that PGPR bacteria *Pseudomonas* strains not only increased tomato and spinach plant shoot dry weight and leaf chlorophyll content in some cultivars, but *Salmonella* microbial activity was reduced in both the spinach and all of the tomato cultivars' phyllosphere (Hsu and Micallef, 2017). However, the effect of the *Pseudomonas* strains showed different levels of plant growth promotion and

Salmonella reduction, which also differed for the plant host species and cultivars. It was theorized that the inoculation of these agricultural plants with *Pseudomonas* may have increased the accumulation of unfavorable metabolites in the plants such as fatty acids and phenolics (Hsu and Micallef 2017). While more research is needed on the effects of PGPR on acting as a biocontrol agent to enteric human pathogens on plants (specifically the antagonistic mechanisms), there are some positive indicators in this research, though ultimately the pairing of the PGPR strain with the enteric human pathogen and the host plant may vary and correct matching may be vital for success.

2.8 Fruit Ripening

During fruit development both auxin and cytokinin have been determined as primary fruit maturation plant hormones with auxin playing the most significant role during fruit maturation (Kumar et al, 2014). As tomato fruits grow, they begin to reach a ripening stage that is triggered by changes in ethylene production in which (1-aminocyclopropane-1-carboxylic acid) ACC is converted to ACC oxidase by the enzyme ACC synthase. When ethylene receptors are degraded along specific genetic targets, transcription of ethylene begins. This is also paired with the MADS box domain genes which induce physiological flower formation (Klee and Giovanni, 2011). The fruit ripening begins with lipid peroxidation and protein oxidation. As catalase activation increases, the tomato fruit will begin to change color more. This is also paired with hydrogen peroxide accumulation. Tomato fruit ripening and color changes occur on another level as the chlorophyll begins to degrade and chloroplasts are converted to chromoplasts to serve as synthesis sites for metabolites such as carotenoids, fatty acids, and amino acids. Changes in gene expression in the biosynthesis of ethylene as well as carotenoids have been

demonstrated, but the transcriptional regulation of carotenoid biosynthesis and chlorophyll degradation is not well understood (Bramley, 2002). Many of the genes regulating the carotenoid biosynthesis pathway are determined to be under the influence of ethylene production levels. During the ripening process, starches are converted to glucose and fructose (Ho et al, 1982). Additionally, ABA (abscisic acid) biosynthesis is crucial in the accumulation of lycopene for fruit coloration during ripening (Bai et al, 2021). Recent studies have also determined the importance of auxin (in addition to ethylene) during the later stages of fruit ripening in terms of the regulation of volatile organic compound (VOC) metabolic pathways (Cruz et al, 2018, Tobaruela et al, 2021). Interestingly, there is an inverse relationship between the sugar levels and malic acid levels in ripening tomato fruits. Measurements have indicated for that plant volatile levels, as well as fatty acids, closely follow the timing of carotenoid development, suggesting a triggering of plant volatiles by carotenoid development in the process of fruiting (Bramley, 2002; Klee and Giovanni, 2011). Changes in other compounds in tomato fruit during ripening include a conversion of starches to sugars, and an increase in organic acids (Kumar et al, 2014) as well as an increase in unsaturated fatty acids C16 and C18 (Palmitic acid and Linolenic acid) (Saini et al, 2017) The pericarp of the tomato begins to shift into a softer state as ripening occurs as well with increased cell wall separation and increased pigmentation linked to increased polygalacturonase activity (Ahrens and Huber 1990, Lazan et al, 1989).

The literature reviewed helps in piecing together known variables to investigate *Salmonella* association on tomato fruit surface washes and the impact of PGPR on the phytochemical profiles. This review has outlined the dangers facing the agriculture and food safety industry in regard to *Salmonella* outbreaks and how *Salmonella* can survive and persist in the phyllosphere.

Studies have also shed light on the intricate balance of favorable and unfavorable metabolites present in plants, especially tomato fruits and which ones have been associated with supporting or inhibiting *Salmonella* growth. Furthermore, this review has investigated the impact of plant growth promoting rhizobacteria on not only tomato plants, but also the impact on modulating phythochemicals and the potential to reduce *Salmonella* association. Lastly, this review discussed how the color and texture qualities of tomato fruits change during the ripening process.

Chapter 3: Phytochemical difference in fruit surface washes of Modern and Heirloom tomato varieties impact *Salmonella enterica* growth

1. Introduction

Enteric pathogen foodborne illness outbreaks of *Salmonella enterica* subsp. e*nterica* have been linked to tomato (*Solanum lycopersicum*) fruits in several countries over recent years (CDC 2005, CDC 2007, Greene et al; 2007). *Salmonella* has been known to persist on the phyllosphere and colonize the interior of tomato plants (Kroupitski et al; 2009, Gu et al; 2011). Most microbial research on *Salmonella* indicates that motility and biofilm production are vital to the adhesion, survivability, and persistence of *Salmonella* microbial populations (Maruzani et al; 2018, Yaron et al; 2014). While *Salmonella* can colonize the interior of tomato plants from the rhizosphere, it would reason that in field scenarios, the enteric human pathogen would more likely contaminate the phyllosphere of tomato plants from contaminated soil due to splash effects (Lee et al; 2019). While there are a multitude of nutrients available in the plant interior that would favor *Salmonella* growth, there are also antimicrobial compounds present in plants that would serve as deterrents (Ferelli Han et al; 2020, Kumar and Micallef 201). Furthermore, there are a multitude of induced and constitutive defenses available from plants that would further diminish *Salmonella* contamination (Zaynab et al; 2019, Wittstock and Gershenzon 2002, Reina-Pinto and Yephremov 2009, Yuan et al; 2021). Previous studies have indicated that while sugars, organic acids, and sugar alcohols were correlated with positive *Salmonella* growth, certain fatty acids were associated with negative microbial association (Han and Micallef 2016). While most studies regarding *Salmonella* and tomato plants have involved modernly available hybrid varieties, there have been a few studies involving heirloom tomato varieties. In one

study, red tomato fruit was shown to be more susceptible to *Salmonella* association compared to green or unripened tomato fruit, larger tomatoes had greater *Salmonella* association than smaller cherry tomato fruit, but for both hybrid modern cultivars and heirloom varieties, fruit color was generally not an important factor in *Salmonella* association. Furthermore, *Salmonella* association was most varied from individual cultivars while field grown tomatoes showed lower ability for *Salmonella* to associate than greenhouse tomato fruits. The same study found similar differences in growth of *Salmonella* with higher association on mature vs immature fruit, and higher association on red pigmented fruit vs varieties that lacked red pigmentation (Marvasi et al; 2014).

While much is known of the relationships between *Salmonella* association and tomato plants, there have been few studies on the phytochemical profile of tomato fruits in their relation to *Salmonella* association, and to date only one study involving heirloom tomato fruits and *Salmonella*. By growing a deeper understanding of the phytochemical profile of tomato fruit surfaces for both modernly available and heirloom fruit cultivars and their relation to *Salmonella* association, we can better prepare means to combat foodborne illness outbreaks at a pre-harvest stage.

In order to investigate the relationship between *Salmonella* and tomato fruit, we evaluated the levels of *Salmonella* growth on fruit surface washes and compared the microbial associations with corresponding total sugar quantifications, citric acid levels, and when possible, fatty acid levels for both modern cultivars and heirloom varieties. The correlations between the microbial association and phytochemical profiles of the different tomato cultivars help to shed light on the complex phyllosphere interactions between tomato fruits and *Salmonella* that could provide

avenues towards alternative *Salmonella*-resistant cultivars being used and genetically bred into the market.

2. Materials and Methods

2.1 Field and Greenhouse Design

During the autumn of 2019, six tomato heirloom varieties (Amish Paste, Black Icicle, Purple BumbleBee, Green Zebra, and White Tomesol) were grown in the UMD Greenhouse Complex. Plants were first started from seed (n=6 plants per cultivar), placed in cell trays with propagation mix in the mist germination rooms until the first set of true leaves appeared, at which point they were transferred into 3 gallon pots and placed in greenhouse conditions of 8 hours of light and 16 hours of dark at 26°C during the day and 14°C at night. The heirloom varieties were pruned to prevent overgrowth and promote fruit production. At peak ripeness, fruits were aseptically harvested using nitrile gloves and sterile Whirlpak bags (Nasco, Wisconsin). Fruits were transferred to the lab, placed in 15°C cold storage rooms if they could not be processed the same day, and then photographed and measured for surface area estimations. Fruits were then placed in 30 mL of 5% methanol/95% DI water and were placed on a shaker for 2 h at 200 rotations per minute (rpm). Halfway through the collection, fruits were rotated to ensure maximum surface area coverage. These washes were then used for microbial assays, and total sugar and citric acid quantification. Preliminary fatty acid analysis in the spring of 2020 showed that only one or two fatty acids could be identified, so the second round of heirloom tomato fruits had an additional second wash of 100% ethanol at 200 rpm in order to collect a more detailed fatty acid profile in addition to the primary 5% methanol wash.

In the summer of 2020, 8 hybrid varieties (Mountain Merit, Mountain Gem, Dixie Red, Red Pride, BHN 589, Mountain Fresh, Charger, and Celebrity) were grown at the UMD wye facility in high tunnels. At peak ripeness, the tomatoes were harvested and subjected to the procedures as described. In the autumn of 2020, a second round of heirloom tomatoes were grown in the UMD greenhouse and separated into two groups, one inoculated with PGPR *Pseudomonas* sp. (Chapter 3) and one control group without PGPR. These greenhouse heirloom varieties were harvested at peak ripeness and transferred to the Plant Science Building where they were immediately processed for 5% methanol surface washes. Modern varieties wash samples n=39 (Mountain Merit n=5, Mountain Gem n=5, Dixie Red n=6, Red Pride n=5, BHN 589 n=4, Mountain Fresh n=5, Charger n=4, and Celebrity n=5). Heirloom varieties wash samples n= 94 (Amish Paste n=14, Black Icicle n=17, Emerald Evergreen n=13, Green Zebra n=13, Purple BumbleBee n=17, White Tomesol n=20).

2.2 **Color and Texture Analysis**

For many of the fruit from the second round (2021), before the initial washes were taken from the fruit, a series of color and texture analyses was performed to investigate any relationship between quality analysis on phytochemistry and microbial *Salmonella* association. First, a colorimeter was connected to the lab computer and a blank was taken with a protected white slide. Then two opposite sides of each tomato were measured in the CIELAB color space where values “L” scaled lightness or luminance (0 being darker/black and 100 being brighter/white), “a” scaled red/green coordinates, “b” scaled yellow/blue coordinates, “C” scaled chroma (distance from the lightness axis where positive is brighter and negative is darker), and “H” scaled hue (angle starts at the +a* axis and is expressed in degrees) (Figure 1) (Hill et al, 1997).

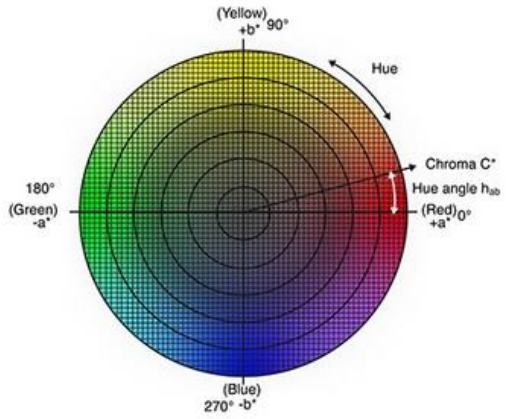

Figure 1. CIELAB color scale (L= light vs dark, a=red vs green, b=yellow/blue, c=bright vs dark, h=angular position around a central or neutral point).

Texture measurements were taken using a fruit texture analyzer in a similar fashion to previous studies involving fruit texture quality analysis (Farcuh et al, 2020, Rolle et al, 2011). A 16 mm probe was standardized using a 2 kg weight. The mechanical fruit texture analyzer was able to calculate stiffness(g/sec), firmness (N), and toughness(g*sec). Single sides of the individual fruits were analyzed for texture analysis to minimize structural damage to the tomato fruit and reduce the risk of puncturing the waxy cuticle surface before surface washes could be collected while color data was taken for two opposite sides of each tomato fruit. Both color and texture data of the individual tomato fruits were averaged across individual fruits for each batch because some sample batches were comprised of individual fruits and some batches were composed of multiple fruits. By averaging the color and texture data across all the individual tomato fruits in the batches, we were able to compare color and texture data to corresponding batch microbial

data and phytochemical data. ANOVA and students t tests were used for color and texture data due to a normal distribution of data. Heirloom varieties color/texture samples n= 33 (Amish Paste n=6, Black Icicle n=9, Emerald Evergreen n=3, Green Zebra n=5, Purple BumbleBee n=5, White Tomesol n=5).

2.3 **Microbial Measurements**

In order to mimic realistic field settings as much as possible, a trio of *Salmonella* strains (*Salmonella* Javiana ATCC BAA-1593; a clinical isolate from a tomato outbreak, *Salmonella Braenderup* 04E61556-2-99; clinical isolate), and and *Salmonella* Newport; a pond water environmental isolate that matched a tomato outbreak strain (Greene et al., 2008) were used to measure the degree to which different tomato fruit surface washes could support *Salmonella* growth and survival. Previous lab *in vitro* settings, *Salmonella* species have commonly been grown on tryptic soy agar (TSA) broth and media. Serial dilutions of suspended *Salmonella* colonies in 0.1% peptone water or 1x PBS solutions have been used to accurately quantify and analyze microbial populations through colony forming unit (CFU) counting (Han and Micallef 2016). From -80°C deep freeze, the three rifampicin-resistant strains were streaked on TSA plates (company, location, country) with rifampicin antibiotic (50µg/ml) (TSA-R) (company, location, coutry) added. After inoculating at 35°C for 20 h, colony forming units were visible. Using a nephelometer (Brand, location, country), 3 separate 10 mL 1x PBS suspensions at an optical density (OD) of McFarland=0.5 were prepared for each *Salmonella* strain using colonies from the fresh culture plates. A series of dilutions were used to enumerate the suspensions where 100ul of the initial 0.5OD solutions were removed and placed in 900ul of PBS before being vortexed and further diluted. At the third dilution, all 3 strains were combined into a 3mL

cocktail in equal volume. This cocktail was further diluted in the same manner two more times to prepare the final inoculum. This was enumerated on TSA-R plates for counting the next day. For inoculation of fruit washes, 100 ul of ~4 logCFU/ml were added to 900 ul of the tomato fruit washes and vortexed for a final inoculum load of ~3 logCFU. A few tubes were plated right away for T0 (time 0 h) counts and the rest incubated at 35°C with shaking at 200 rpm for a 20 h (T20) count. The following day, the fruit wash suspensions went through three serial dilutions at which point all 3 had 20 ul plated and incubated at 35 ^{o}C for 20 h before colony count recovery occurred the following day. The colony counts and corresponding back calculations allowed us to accurately quantify the ability of the tomato fruit surface washes to support the *Salmonella* strains.

2.4 **Total Sugar Quantifications**

Using the tomato fruit washes, we followed a total sugar quantification protocol using a spectrophotometer (Cuesta et al; 2003). Aliquots of 100ul of the surface washes were mixed with 100 ul of 5% phenol solution (brand, location, country) and 500ul of concentrated sulfuric acid (brand, location, country). The centrifuge tubes were then heated at 100 ^{o} C for 5min and then vortexed before being read in the spectrophotometer (brand/model, location, country) at 490nm. Sucrose stocks and blanks were used to establish a standard curve. Total sucrose concentration was read as mg sucrose/ml of wash. Measurements of the total sugar quantifications were analyzed by cultivar and against the microbial data. When evaluating sugar values, similarly to the microbial analysis, comparisons were made via t test one way analysis between the modern and heirloom varieties, but wilcoxon each pair tests were made within both the modern and heirloom cultivars due to an uneven distribution of data points.

2.5 **Citric Acid Measurements**

The citric acid content of the surface fruit washes was measured using [Megazyme citric acid assay] (Megazyme, Cork, Ireland) following the microplay assay protocols. First, the hydrated solutions were prepared according to the instructions in the protocol. Then, a stock solution of solutions 1, 2, and 3 (buffer solution, NADH/PVP solution, and L-MDH/D-LDH) was created so that a consistent 72 ul could be added to each well plate and reduce variation in the smaller volumes being used. 20ul of the fruit washes were added to 180ul of DI water (while 200ul water was used as the blank and 20ul standard solution and 180ul DI water was used to make the standard solution). After the addition of the stock solution mix to each sample, blank, and standard well plate, at which point spectrophotometer readings were taken on a Synergy microplate reader at 340nm. The mixtures were incubated at 37°C for 4 mins, then 2ul of the enzyme solution 4 was added to each well and mixed before taking an additional reading. 3rd and sometimes 4th readings were taken while continuing to incubate at 37degC for 2 mins in-between readings to ensure that the reactions had all taken place between the first and second readings. Using the change in standard well reading and any present dilution factors, measurements were recorded in mg/ml.

2.6 **Fatty Acids**

For investigating the fatty acid profiles of the fruit washes, we used a fatty acid methyl esterification technique (FAME) and gas chromatography mass spectrometry (GC-MS) on an Agilent 6890N GC system coupled with a JEOL Mstation magnetic sector mass spectrometer. The fatty acids in the 100% ethanol fruit washes were derivatized by ester derivatization as

described. Once dried, the metabolites would be suspended in 500ul of hydrochloric [SM2] acid in methanol and heated at 78℃ for one hour before having 500ul of 99% hexane added. Then 5ml of calcium carbonate solution was added and vortexed. The solutions then went through micro centrifuge at 1000rpm for 10 mins and the hexane layer was recovered. Another 500ul of hexane was added again and the samples centrifuged again before the next layer of hexane was added to the initial recovered layer. The hexane-metabolite mixes were dried [SM3] and suspended in 125ul of methanol with 10ul of methyl octanoate (TCI, Japan) as an internal standard added. Samples were run in the GCMS and spectra read outs were compared to NIST database and sample standards[SM4] methyl myristate (TCI, Japan), methyl palmitate (Sigma, Malaysia), methyl linoleate (arcos organics, USA), methyl linolenate (arcos organics, USA), methyl stearate (Alfa Aesar, USA), methyl erucate (Restek, USA), methyl arachidate (Alfa Aesar, China). Areas under the curve were calculated relative to the area under the internal standard curve and calibrated with respect to fruit surface areas for statistical analysis. The fatty acid methyl ester GCMS analysis was done for the heirloom variety cultivars. Peak area under the curve for each spectra was corrected for relative internal methyl octanoate standard followed by a correction for each sample batch fruit surface area. In comparing relative fatty acid levels, Wilcoxon each pair tests were used due to an uneven distribution of data. The gas chromatography measurements were performed on an Agilent 6890N system coupled with a JEOL high-resolution magnetic sector mass spectrometer (JMS-700 MStation) with the EI ion source (70 eV). The mass spectrometer was operated in the mode of high scan speed and low resolution (1000) with the mass range from 50 to 500 daltons. A silica capillary column (Agilent DB-5, 60 m length, 250 μm I.D.) was used with helium (at 1 ml/min) as the carrier gas. Analysis was performed as follows: injection volume was 1 μl, the inlet temperature was 285 ℃ in split

mode (split ratio = 2), the column temperature was programmed from 70 °C at 3.0 min, then increased to 310 °C at the rate of 15 °C/min and then held at 310 °C for another 6.0 minutes. Heirloom varieties fatty acid samples n= 54 (Amish Paste n=8, Black Icicle n=13, Emerald Evergreen n=9, Green Zebra n=5, Purple BumbleBee n=9, White Tomesol n=10).

2.7 Statistical Analysis

When looking at the microbial counts from the T0 and T20 points, the change (delta) in log colony forming units(CFU)/ml, was used to distinguish the degree of microbial association with the surface washes. Additionally, microbial counts were adjusted in regards to relative surface area of the tomato fruits. When comparing modern and heirloom varieties in terms of *Salmonella* association, we used a t test to compare means. Evenly distributed data was analyzed using students t test while uneven distribution of data was analyzed using Wilcoxon each pair test. Regression analysis was used to determine the degree of influence of variables on *Salmonella* association and the correlations with microbial counts. All data was analyzed using JMP pro15 2.0.

3. Results

3.1 Microbial Analysis

Surface washes from fruit of modern cultivars were in general better able to support modern *Salmonella* growth (average count = 2.0 delta log/CFU/ml) than washes from heirloom fruit (average count = 1.7 delta log/CFU/ml) but this difference was only weakly significantly different (p=0.06) (Figure 2).

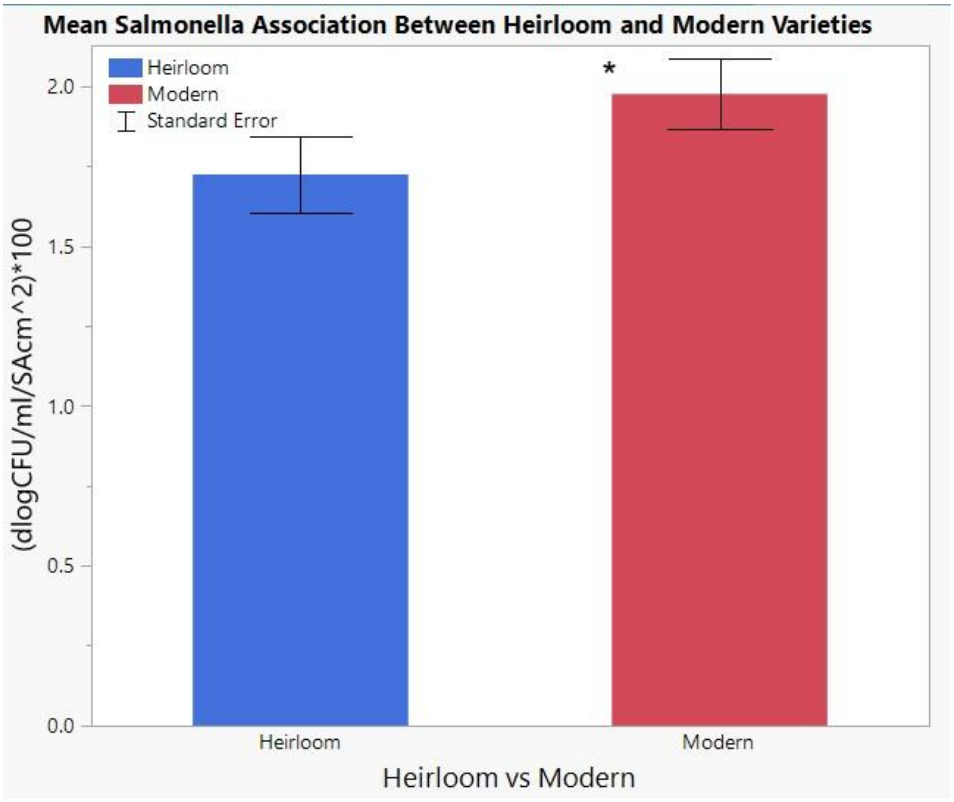

Figure 2. Analysis of *Salmonella* growth on exudate washes of Heirloom and Modern varieties. Error bars indicate stnd error. Asterisk indicates significant differences. (p=0.06)

Using *a priori* multiple comparisons, no statistical difference in *Salmonella* counts were detected among the modern cultivars. 'Dixie Red' and 'Red Pride' supported the highest *Salmonella* populations, with delta logCFU differences between T20 and T0 of 2.3 and 2.2 log/CFU/ml, respectively. On the other hand, 'Mountain Gem' and 'Charger' supported the least growth, with 1.7 ΔlogCFU/ml values modern(Figure 2). When analyzing the heirloom varieties, cultivar 'Emerald Evergreen' fruit washes supported the highest population of *Salmonella* (2.6

ΔlogCFU/ml) and was statistically different from all other heirloom cultivars (p<0.05) except for 'Amish Paste' (Figure 3). In fact, 'Emerald Evergreen' provided the most favorable solution for *Salmonella* growth overall and was comparable to the modern cultivar 'Dixie Red'. Comparing all cultivars together revealed that 'Dixie Red' was also different from 'Purple Bumblebee' and 'Black Icicle'. Excluding 'Emerald Evergreen' from the heirloom group revealed a strong difference between modern and heirloom fruit surface washes in their ability to support *Salmonella* (p=0.005) (figure 3).

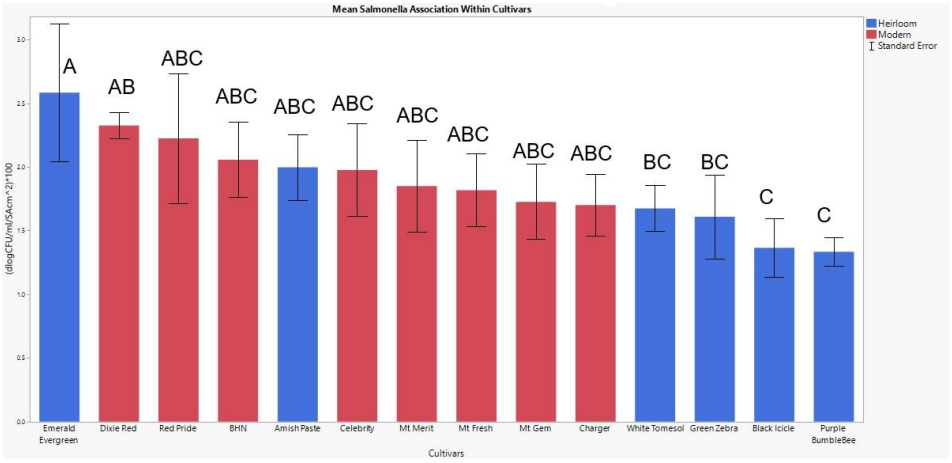

Figure 3. Analysis of *Salmonella* growth on fruit surface washes within Heirloom and Modern varieties. Error bars indicate stnd error. Letters denote significant differences. (p<0.05)

3.2 **Color and Texture Analysis**

While none of the field modern varieties were analyzed for color and texture, the second round of greenhouse heirloom varieties were analyzed for color and texture. In terms of "L" scaling (light vs dark), cultivar white tomesol (WT) showed the lightest color followed by green zebra

(GZ), then both purple bumblebee (PBB) and emerald evergreen (EE), then amish paste (AP), and finally black icicle (BI) as the darkest. For "C" chroma scaling (bright vs dull), cultivars amish paste, and green zebra showed the highest chroma followed by emerald evergreen, black icicle, purple bumblebee, and white tomesol. For "h" hue (a combination scaling of "a" and "b"), cultivar white tomesol (WT) showed the highest hue followed by GZ, EE, PBB, BI and AP as the lowest hue cultivars ($p<0.05$) (Figure 4).

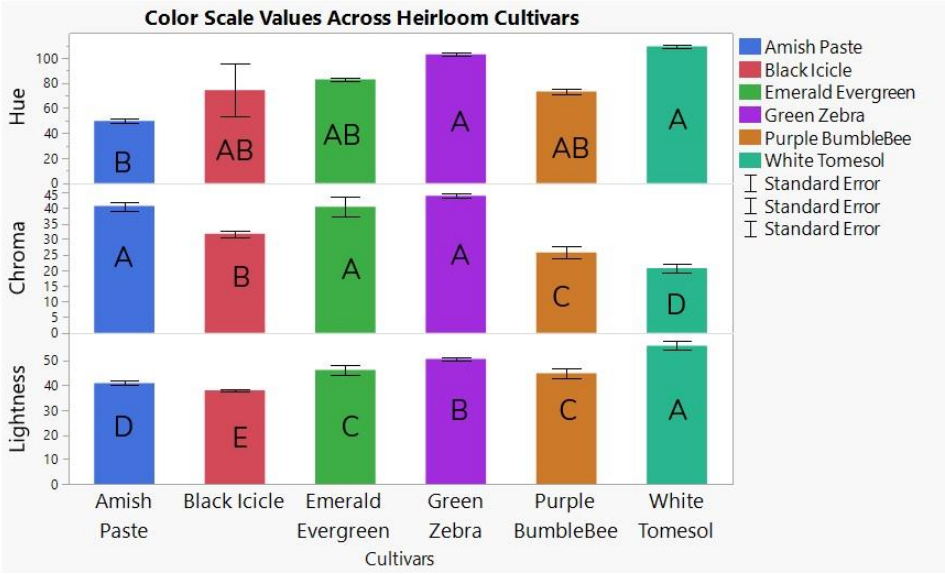

Figure 4. Analysis of color scale values by cultivar for Heirloom varieties. Error bars indicate stnd error. Letters indicate significant differences. ($p<0.05$)

For texture analysis, data was analyzed using ANOVA and student's t test. There were no significant differences detected between the heirloom cultivars in terms of stiffness. However, for both toughness and firmness, cultivar PBB demonstrated the highest values while cultivars AP, BI, and WT demonstrated the lowest toughness and firmness values ($p<0.05$) (Figure 5).

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Stiffness was measured in g/sec, firmness was measured in g, and toughness was measured in g*sec.

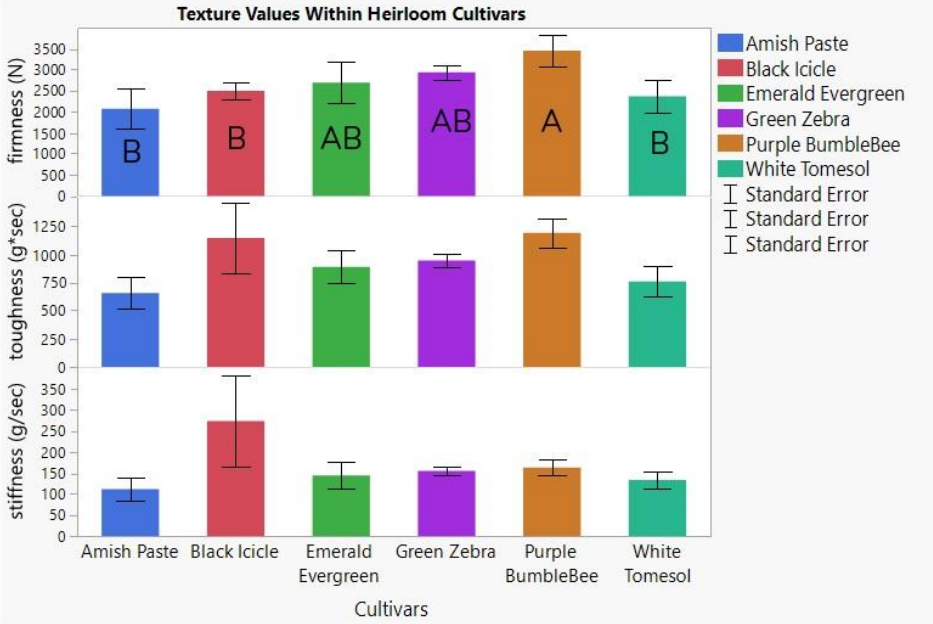


Figure 5. Analysis of texture values by cultivar within Heirloom varieties. Error bars indicate stnd error. Letters indicate significant differences. ($p<0.05$)

3.3 **Sugar Analysis**

Overall, heirloom varieties had a higher level of total sugars (25.1 g/100 ml) compared to modern varieties (6.2 g/100 ml) ($p<0.001$) (Figure 6), even when 'Emerald Evergreen' was excluded (24.5 g/100 ml; $p<0.001$)

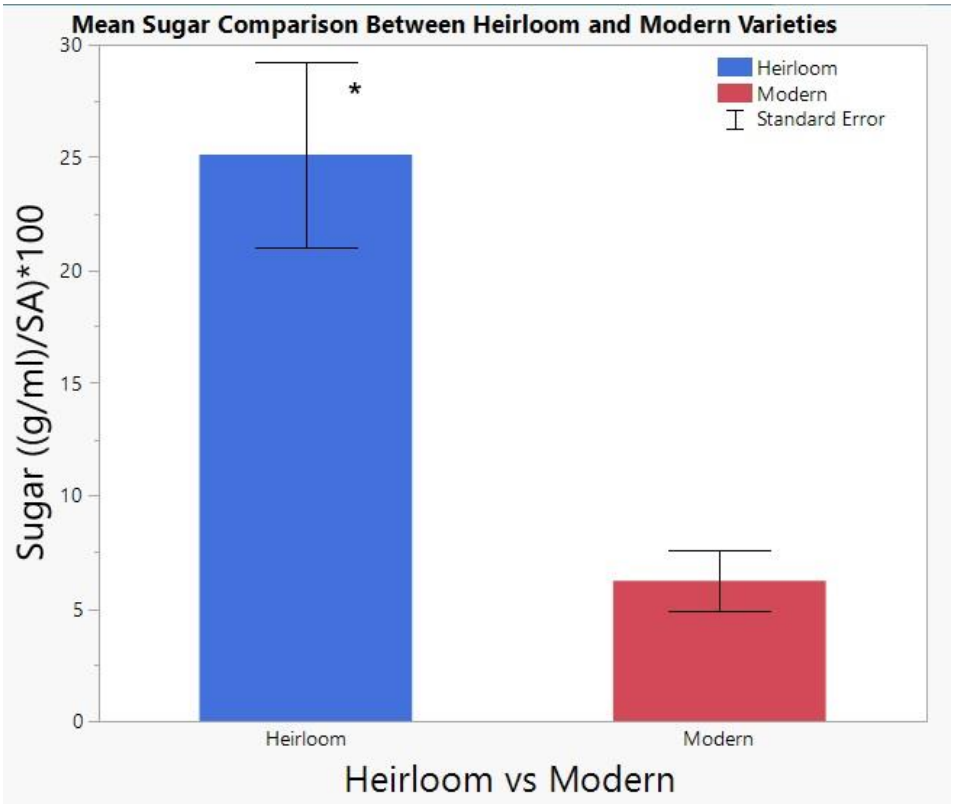


Figure 6. Analysis of total sugar quantification between Heirloom and Modern varieties. Error bars indicate stnd error. Asterisk indicates significant difference. ($p<0.05$)

Within the modern cultivar group, significantly different sugar values were detected between 'Mountain Merit' having higher total sugar values than cultivar 'Charger' ($p<0.05$). However, cultivars 'Dixie Red' and 'Charger' approached significance ($p=0.07$) (Figure 7). Within the heirloom varieties, cultivars PBB and EE approached significant difference ($p=0.07$) however none of the cultivar sugar quantification differences managed to reach statistically different values ($p<0.05$) (Figure 7). Comparing the modern cultivars with the heirloom varieties revealed

several differences. While total sugars varied more in modern cultivars compared to heirloom cultivars, the modern cultivars demonstrated overall lower total sugars compared to the heirloom cultivars (Figure 7). Modern cultivar Mt Gem supported some of the least *Salmonella* growth among modern cultivars and yet has highest sugars among the modern cultivars. Heirloom cultivar EE has similar total sugars as most other heirloom cultivars and yet is more favorable to *Salmonella*. Meanwhile, heirloom cultivar PBB is lowest for bacterial growth, but not the lowest for sugars when compared to modern cultivars. (Figure 7).

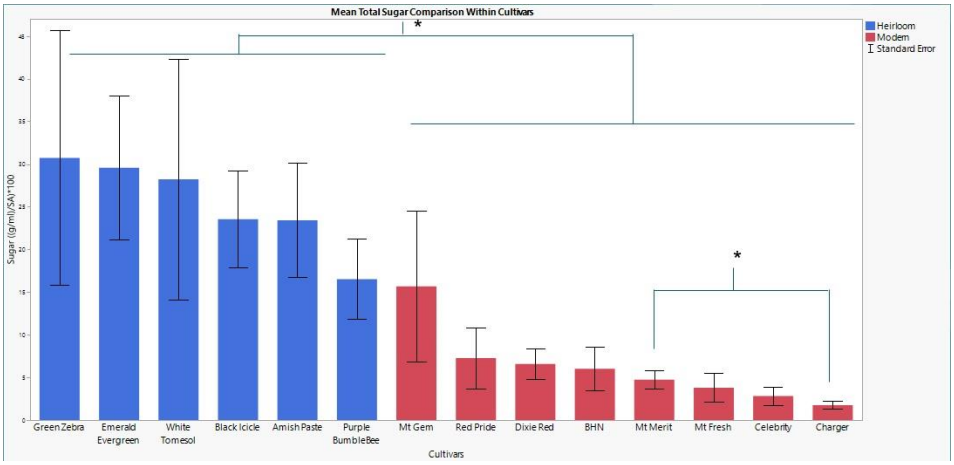


Figure 7. Analysis of total sugar quantification within cultivars. Error bars indicate stnd error. Asterisks indicate significant differences. ($p<0.05$)

3.4 **Citric Acid Analysis**

Another component investigated in this experiment was the level of citric acid in the fruit washes and the correlation with *Salmonella* association. A non-parametric test was used for comparing modern and heirloom averages while wilcoxon each pair tests were used for comparisons within the cultivars of each variety. In the initial comparison between modern and heirloom varieties, it

was seen that heirloom varieties had a weakly statistically significant higher amount of citric acid compared to the modern varieties (p=0.098) (Figure 8).

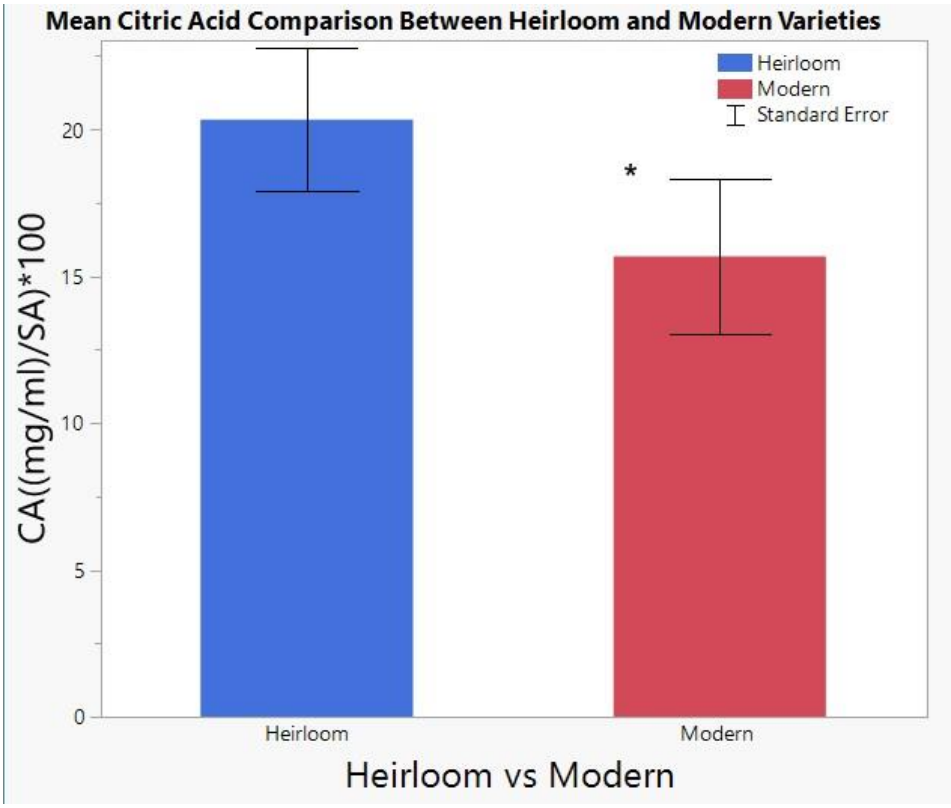


Figure 8. Analysis of citric acid across heirloom and modern varieties. Error bars indicate stnd error. Asterisk indicates significant difference. (p=0.098)

Meanwhile, within modern cultivars, Mt. Merit had the highest level of citric acid while cultivars Charger, Celebrity, and Mt. Fresh had the lowest citric acid levels ($p<0.05$) (Figure 10). For the heirloom varieties, cultivar EE demonstrated the highest citric acid levels while BI and PBB demonstrated the two lowest citric acid levels ($p<0.05$) (Figure 10). When comparing the

modern and heirloom varieties, the modern cvs. ‘Mountain Merit’, Mountain Gem’ and BHN# 589’ had higher citric acid levels than the heirloom 'Purple Bumblebee’ (p<0.01). On the other hand, the heirloom variety ‘Emerald Evergreen’ had higher citric acid levels than the modern cvs. of ‘Mountain Fresh’ (p<0.01), ‘Celebrity and ‘Charger’ (p<0.05) (Figure 9).

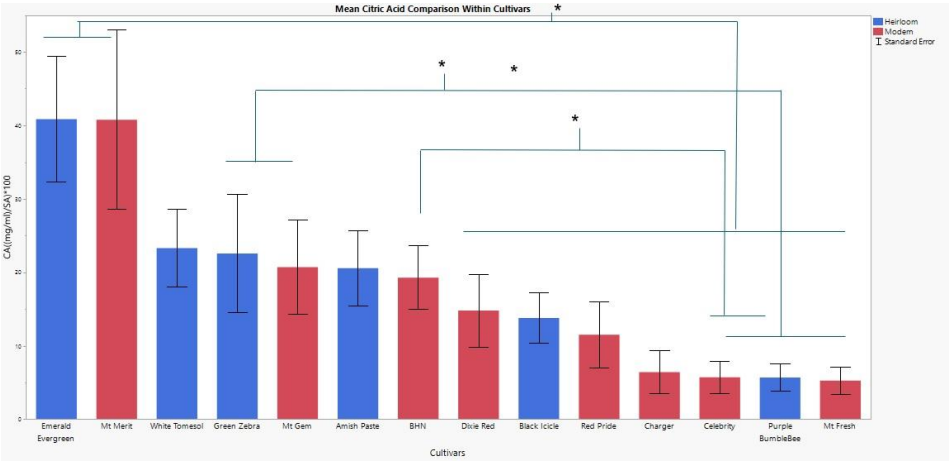


Figure 9. Analysis of citric acid within cultivars. Error bars indicate stnd error. Asterisks indicate significant differences. (p<0.05)

3.5 **Fatty Acid Analysis**

There were seven main fatty acids peaks identified using standards in the spectra. Four of these were saturated fatty acids: arachidic acid (eicosanoic acid, C20:0), stearic acid (octadecanoic acid, C18:0), palmitic acid (hexadecanoic acid, C16:0), myristic acid (tetradecanoic acid, C14:0), and three were unsaturated: linoleic acid (C18:2 cis-9,12), linolenic acid (C18:3 cis-9,12,15), erucic acid (docosenoic acid, C22:1 cis-13).

In general, cultivars EE and AP had the lowest levels of saturated fatty acids while BI, PBB and WT had the highest. Our data showed that stearic acid and hexadecanoic acid were higher in cultivars WT, PBB, and BI ($p<0.05$) than cultivars AP and EE. Meanwhile cultivars BI, PBB, and WT had higher myristic acid ($p<0.05$) and compared to cultivar AP. To a lesser degree, myristic acid was also somewhat lower in GZ and ($p<0.1$) compared to AP. WT and BI had slightly higher levels of hexadecanoic acid (palmitic acid) than GZ ($p<0.1$). Additionally, for arachidic acid, cultivar WT had higher levels compared to cultivar EE ($p<0.05$) (Figure 10).

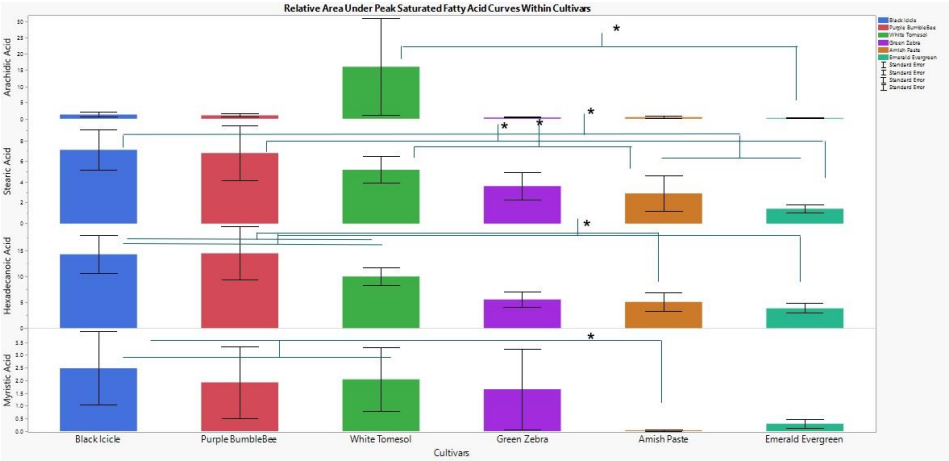


Figure 10. Analysis of saturated fatty acids. Error bars indicate stnd error. Asterisks indicate significant differences. ($p<0.05$)

Fewer cultivar differences were detected for polyunsaturated fatty acids (linoleic and linolenic acids). Our data showed that cultivars PBB, WT ($p<0.05$) and BI ($p<0.1$) had higher levels of linoleic acid compared to cultivar EE. Also, cultivars PBB, WT, and BI had higher levels of linolenic acid compared to cultivar AP ($p<0.05$). Some other weaker differences were for augmented levels of linolenic and linoleic acids in PBB compared to GZ ($p<0.1$) (Figure 11).

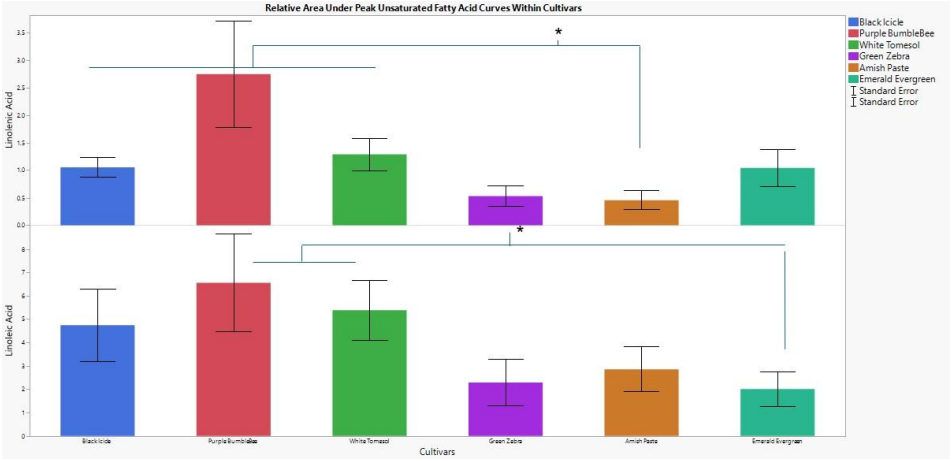


Figure 11. Analysis of unsaturated fatty acids. Error bars indicate stnd error. Letters indicate significant differences. ($p<0.05$)

The only monounsaturated fatty acid detected in the GCMS spectra for our fruit washes was erucic acid. In our analysis, PBB, BI, WT ($p<0.01$) and GZ ($p<0.05$) had more erucic acid compared to cultivar EE (Figure 12). Slightly higher levels of erucic acid were also detected in PBB than AP ($p<0.1$).

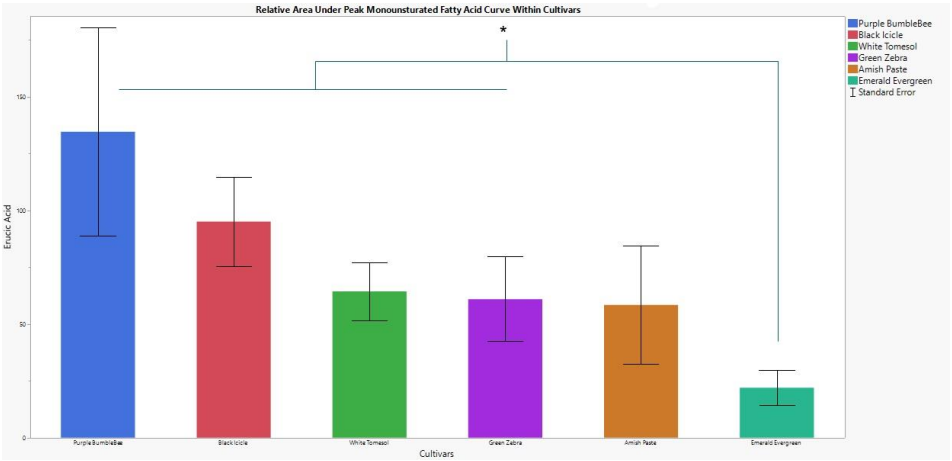


Figure 12. Analysis of monounsaturated fatty acids. Error bars indicate stnd error. Asterisks indicate significant differences. ($p<0.05$)

In general, cultivars EE and AP had the lowest levels of saturated fatty acids while BI, PBB and WT had the highest. Our data showed that stearic acid and hexadecanoic acid were higher in cultivars WT, PBB, and BI ($p<0.05$) than cultivars AP and EE. Meanwhile cultivars BI, PBB, and WT .

3.6. Relationship between phytochemicals in fruit and *Salmonella* growth

To assess whether sugar and citric acid or fatty acid levels in heirloom compared to modern tomato fruit washes could significantly predict *Salmonella* growth in fruit washes, multiple regression was employed. The results of the regression using sugar and citric acid values as predictors for modern varieties showed that the model explained 6% of variance significantly ($R^2=0.6$, $p<0.001$). Citric acid ($p<0.01$) and sugars ($p<0.05$) were positively correlated with *Salmonella* growth for modern varieties. For the heirloom regression model using citric acid and sugars as predictors, the model showed that 21% of the variance of *Salmonella* could be explained, but was not significant ($R^2=0.21$, $p=0.31$) with neither citric acid ($p=0.17$) nor sugars ($p=0.38$) showing significant contributions to *Salmonella* variance (Appendix Table 1).

4. Discussion and Conclusion

4.1 Microbial-Sugar-Citric Acid Analysis Discussion

The purpose of our study was to investigate the color, texture, and phytochemical profiles of heirloom and common variety tomato fruit surfaces and to analyze correlations of these profiles to *Salmonella* association in order to better understand the relationships between tomato fruit

surfaces and potential *Salmonella* association to pave a path for the reduction of Salmonella at a pre-harvest stage. Firstly, the main comparison was between Modern and Heirloom variety tomato fruit phytochemical profiles. In order to both mimic realistic surface contact scenarios with *Salmonella* from the soil and to capture phytochemical metabolites from the tomato surface, we both grew *Salmonella* and chemically analyzed tomato fruit surface washes. From our results, there are several components that contribute to the bacterial growth of *Salmonella* on the tomato fruit washes. For the modern varieties, although there was only a weakly significant difference in microbial association, there were significant differences in sugar levels and weakly significant differences in citric acid levels between modern and heirloom cultivars. The cultivar EE demonstrated the highest *Salmonella* association as well as some of the highest sugars and citric acid levels while cultivar Charger demonstrated some of the lowest *Salmonella* association and some of the lowest sugars and citric acid levels. *Salmonella* has been known to utilize glucose as a major carbon source (Bowden et al; 2009), but tomato fruits are known to contain glucose, fructose, and galactose (Zhao et al; 2016), and our method of total sugar quantification only measured total sugars instead of individual sugars. Furthermore, heirloom varieties as a whole had higher sugar and citric acid levels than modern varieties, yet in our regression analysis, only in the heirloom cultivars did sugars and citric acid levels significantly contribute to *Salmonella* growth distribution and demonstrate positive correlation with microbial counts. We might expect that the heirloom varieties would have higher levels of *Salmonella* association than modern varieties due to their higher sugars and citric acid levels, but this did not appear to be true. While citric acid at high levels are known to have anti-microbial properties (Al-Rousan et al; 2018) it is possible that the surfaces washes did not contain high enough levels to demonstrate anti-microbial effects, and it is also possible that *Salmonella* could utilize citric acid in a different

state as an energy source as citrate with the help of 2-methylcitrate synthase (Horswill and Escalante-Semerena 1999). Notably, the regression model even for the heirloom cultivars when including only sugars and citric acids could explain only 21% of the microbial count distribution, suggesting that for both heirloom and modern cultivars, other metabolites have a more significant impact on *Salmonella* association. Furthermore, cultivar PBB had higher toughness and firmness compared to cultivar AP, suggesting that the toughness and firmness of the waxy cuticle could play a part in the releasing of favorable phytochemical metabolites that could support *Salmonella* growth.

There are also a number of additional compounds that were not analyzed in these experiments that could be deterring microbial growth. When looking at the heirloom varieties, there appears to be a larger degree of variation within the cultivars compared to the modern cultivars. Studies done on the genetic diversity of cultivated tomato fruits have indicated a large dip in the 1960s followed by a significant increase in recent decades. It is also known that older heirloom varieties have a higher nutritional value compared to modern cultivars and could potentially be used in modern breeding varieties to increase nutritional value (Schouten et al; 2019) (Fita et al; 2015) (Zsogon et al; 2018). While there is still debate on the degree to which modern breeding has led to a loss in genetic variation and in what specific ways, our research has indicated at least a wider variation in phytochemical profile of the tomato surface in heirloom varieties for *Salmonella* growth compared to modern varieties. Some of these variations could be correlated with differences in genetic variation for the comparison between these specific heirloom and

modern cultivars that were investigated. It is possible that other heirloom and other modern cultivars would have less variation as well.

4.2 Fatty Acids Discussion

Previous studies have identified medium chain fatty acids as correlated with reduced *Salmonella* association (Han and Micallef 2016), but this research was conducted on modern cultivars when we instead investigated heirloom cultivars. The saturated fatty acids (myristic acid, hexadecanoic acid, stearic acid, and arachidic acid) data showed that cultivars BI, PBB, and WT had higher myristic acid compared to cultivar AP. Cultivars PBB, WT, and BI also had higher hexadecanoic acid and stearic acid compared to cultivars AP and EE. Additionally, for arachidic acid, cultivar WT had higher levels compared to cultivar EE. Similar trends were seen with cultivars PBB and BI having some of the highest unsaturated fatty acids compared to cultivar EE. Similarly, cultivar PBB had higher erucic acid compared to cultivar EE. Much of this is in line with the differences seen in the microbial growth for the heirloom cultivars, but no negative correlations are seen. The similarities in the differences between the microbial growth and several of the fatty acids, especially for cultivars EE vs PBB (the two most extreme), show that fatty acids might play a role in deterring *Salmonella* growth, but ultimately it appears to depend upon the cultivar. Fatty acids and color/texture values were included in chapter 4 regression models for comparing PGPR+/- groups as chapter 3 was focused on comparing heirloom and modern cultivars and only sugars and citric acid levels were measured for the modern cultivars. Previous studies have shown antimicrobial properties from certain fatty acids either through the disruption of bacterial cell membranes (Ricke 2003) or through the reduction in activation of *Salmonella* pathogenicity genes (Van Immerseel et al, 2004). Interestingly, previous studies also

showed linolenic acid being negatively correlated with *Salmonella* growth (Han and Micallef 2016), which is in line with cultivars demonstrating higher linolenic acid also on average having lower *Salmonella* association.

4.3 Discussion Conclusion

Overall, it appears there are multiple factors that go into promoting *Salmonella* growth like sugars, citric acid, and low toughness and firmness while certain factors can also deter *Salmonella* growth such as low sugars, low citric acid levels, higher levels of certain fatty acids, and high toughness and firmness. It would reason that having higher toughness and firmness can limit *Salmonella* from having entry points into the fruit interior in a field scenario, and it also would reason that having high levels of toughness and firmness can reduce the release of favorable metabolites. Ultimately, being able to deter *Salmonella* growth on tomato fruit surfaces seems to be a complex balance between a lack of favorable metabolites and a greater level of unfavorable metabolites that differs between cultivars and between heirloom vs modern tomato varieties. It is important to keep in mind that many metabolites were not analyzed in these experiments that could shed more light on *Salmonella* association with tomato fruits. Some of the most notable anti-microbial metabolites that could be playing a factor are volatile organic compounds (VOCs) such as terpenoids, phenolics, and flavonoids (Schulz-Bohm; et al 2017, Tako et al; 2020). Future studies should investigate these compounds in modern and heirloom varieties and their relationship with *Salmonella* association.

Chapter 4: Inoculation of Solanum lycopersicum (tomato) plants with plant-growth promoting rhizobacteria *Pseudomonas* sp. (S4)

1. Introduction

In this chapter, we investigated the impact of tomato root inoculation of PGPR *Pseudomonas* sp. on the phyllosphere, specifically the phytochemical profile change of the heirloom tomato fruits and the potential mitigation of *Salmonella* association. Plant-growth promoting rhizobacteria (PGPR) are a class of beneficial bacteria that engage in a mutualistic, symbiotic relationship with a wide range of plants. Many of the impacts of PGPR that have been studied are focused on the impacts of induced systemic resistance (ISR) against biotic pathogens, to prime plants against future abiotic stresses such as drought and salinity (Beneduzi et al; 2012, Bhattacharyya and Jha 2012, Arora et al; 2011). After engaging in a mutualistic relationship with PGPR, microbes such as *Bacillus* and *Pseudomonas* aid plants in nitrogen fixation, plant growth promotion, plant hormone synthesis, and aid in resistance against plant pathogens (Sivasakthi at al; 2014). PGPR including *Bacillus* have also been known to help plants in resistance to abiotic stress such as salinity, drought, and heavy metals in potato plants (Gururani et al; 2013). There is also some research on the impacts of PGPR with the nutritional quality of plant produce. Some studies have shown PGPR (many including *Pseudomonas*) to increase vitamin quantity lycopene content, and texture in tomato fruit (Loganathan at al; 2014, Sharafzadeh et al; 2012, Mena-Violante et al; 2007). For tomato plants in particular, one of the most commonly used PGPR bacterial strains is *Pseudomonas* sp. which has been shown to increase shoot dry weight, antioxidant and lycopene levels, and reduce negative impacts from phytopathogens such as *Spodoptera litura* by means of proline and polyphenol production (Bano and Muqarab 2017). Interestingly, more beneficial impacts of PGPR *Pseudomonas* sp. were seen in tomato

plants when co-inoculated with other PGPR strains of the same genus, suggesting potential symbiosis and synergism between bacterial PGPR strains (Almaghrabi et al; 2013, Ordookhani1 et al; 2010).

. As previously described in the literature review, there have been a few studies indicating limited success with PGPR in mitigating enteric human pathogens on plants (Markland et al; 2015, Allard et al; 2014), and there has only been one study on using *Pseudomonas* to lessen the association of *Salmonella* with tomato plants (Hsu and Micallef 2016). It is possible that the inoculation of tomato rhizosphere with PGPR *Pseudomonas* could induce a systemic immune response that upregulates unfavorable metabolites for *Salmonella* and possibly downregulate favorable metabolites for *Salmonella* to reduce the association and potentially provide an avenue for reduced foodborne illness outbreaks with the use of PGPR in pre-harvest agriculture stages. In this study, we aimed to determine if the inoculation of tomato roots with PGPR *Pseudomonas* sp. can modulate the phytochemical profile of tomato fruit surfaces to be unfavorable to *Salmonella* growth and subsequently limit *Salmonella* growth. There in this study we aimed to analyze whether or not the inoculation of heirloom tomato plants with PGPR *Pseudomonas* sp. would be able to modulate the phytochemical profile of the surface of the heirloom tomato fruits and inhibit *Salmonella* association.

2. Materials and Methods

2.1 Greenhouse Design and PGPR Inoculation

Similarly to the heirloom variety tomato plants outlined in chapter 3, six heirloom tomato cultivars Amish Paste (AP), Black Icicle (BI), Emerald Evergreen (EE), Green Zebra (GZ), Purple Bumble Bee (PBB), and White Tomesol (WT), were planted in the UMD Greenhouse

Research Complex. In the autumn of 2019, only non-PGPR tomato plants were considered, but in the autumn of 2020, two new groups were introduced. Of each cultivar, four PGPR non-inoculated and four PGPR-inoculated plants were divided once transferred from the mist germination room to the larger quart pots. Growing conditions were set at 8 hours of light and 16 hours of dark at 26 °C during the day and 14 °C at night. Rifampicin (rif) resistant *Pseudomonas* sp. (unconfirmed to be species spp., so classified as *Pseudomonas* sp.) strain S4 (Dr. Brain Klubek (Southern Illinois University Carbondale) has been previously kept in our lab. Frozen stock of *Pseudomonas* sp. strains were streaked on Trypticase Soy Agar (TSA) plates and incubated at 30°C for 48 hours. Single colonies were transferred to Trypticase Soy Broth (TSB) and grown to late-log phase. Suspensions were incubated at 200pm, 30deg C for 20 hours before being pelletized at 10,000 rpm for 15 min and diluted with 1x PBS to OD=0.5. Tomato plants received 2 mL of 10^8 CFU/mL bacterial suspension or 1xPBS at the base of stems. Two separate root inoculations were carried out 2-days and 9-days post re-potting. Successful root colonization with each strain of *Pseudomonas* was confirmed by colony enumeration of root rinsates on TSA-rif plates upon completion of experiments. the heirloom varieties were pruned to prevent overgrowth and promote fruit production. At peak ripeness, fruits were harvested using nitrile gloves and sterile whirl pak bags to maintain sterility. Fruits were taken to the UMD PLSC Plant Science building, and in the food safety lab then had their pictures taken to account for surface area later on. Fruits were first placed in 30mL of 5% methanol/95% DI water and were placed on a shaker for 2 hours at 200 rotations per minute (rpm). Halfway through the collection, fruits were rotated to ensure maximum surface area coverage. After the initial wash, the tomato fruits were dried and transferred to new whirl pak bags in a second 100% ethanol wash at 200rpm for 2 hours in order to maximize the collection of the fatty acid profiles

for the tomato fruit surfaces. PGPR+ group surface wash samples n= 57 (Amish Paste n=12, Black Icicle n=10, Emerald Evergreen n=8, Green Zebra n=11, Purple BumbleBee n=9, White Tomesol n=7). PGPR+ group color/texture samples n= 43 (Amish Paste n=7, Black Icicle n=9, Emerald Evergreen n=7, Green Zebra n=7, Purple BumbleBee n=6, White Tomesol n=7).

2.2 Plant Growth Promotion Analysis

Two additional investigations were done for the comparison between PGPR+ (the group of plants inoculated with PGPR) and PGPR- (the group of plants not inoculated with PGPR). The first was simply to measure for plant growth promotion and the second was to analyze for leaf chlorophyll analysis. In order to test for plant growth promotion, plants were harvested just before flower formation began (approximately 6 weeks after planting). Tomato plants were cut directly at the base of the stem and wrapped in pre-weighed aluminum foil to minimize water loss. Then plant samples were weighed with both the foil wrappings and the plant samples together. Samples and their assigned foil wrappings were then placed in large ovens in the plant science building and dried at 70^0 C for 20 hours before finally being weighed once more in dried plant samples with their respective foil wrappings. This allowed us to calculate the final fresh weights and dry weights of each sample.

2.3 Additional Methods and Materials Notes

The methods and materials for the tomato growth conditions (Chapter 3 section 2.1), microbial analysis (Chpt. 3, 2.3), color/texture analysis (Chpt. 3, 2.2), total sugar quantification (Chpt. 3, 2.4), fatty acid GCMS analysis (Chpt. 3, 2.6), and citric acid (Chpt. 3, 2.5) analysis for both the non-PGPR inoculated group and the PGPR-inoculated group remained the same as in Chapter 3.

3. Results

3.1 Fresh Weight Dry Weight Analysis

Prior to fresh weight and dry weight growth promotion analysis, *Pseudomonas* PGPR establishment in the rhizosphere of the PGPR+ plant group was confirmed by recording *Pseudomonas* colony growth on TSA-rif plates in the tomato root rinses. Although there was no significant difference between the PGPR+ and PGPR- dry weight across all cultivars, the difference approached significance (p= 0.085). For fresh weight analysis, there was a significant difference between the two groups which the inoculated group had higher fresh weight (p<0.01) (Figure 13).

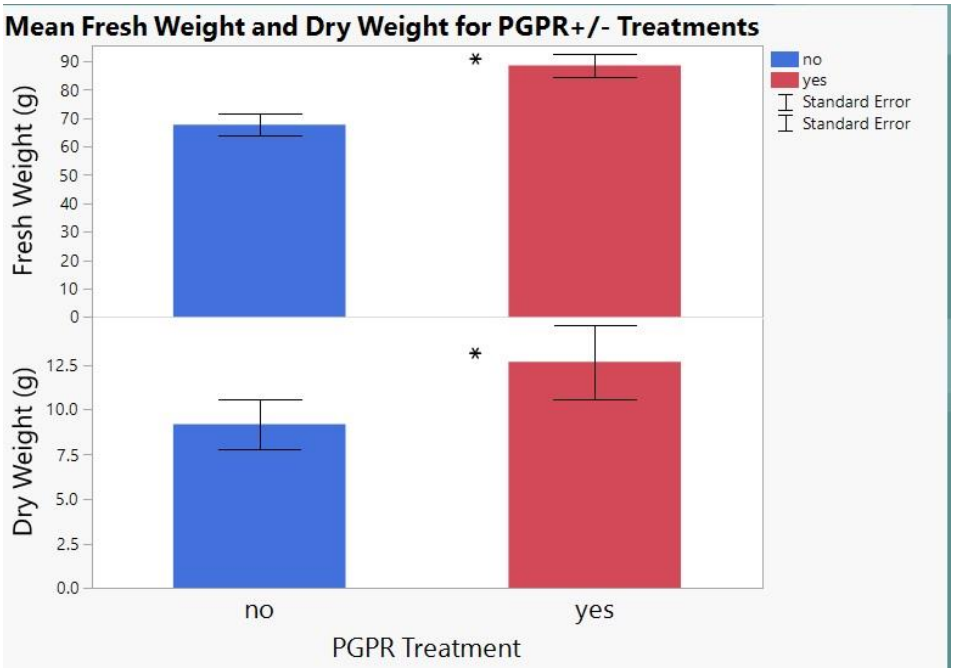


Figure 13. Average fresh weight and dry weight of heirloom plants inoculated (PGPR+) and uninoculated (PGPR-) with PGPR *Pseudomonas* sp. at 6 weeks post-germination. Error bars indicate stnd error. Asterisks indicates significant difference. (p</=0.0857)

When looking at the individual cultivars, rhizosphere colonization with PGPR appeared to promote plant growth as indicated by fresh weights of cultivars AP, EE, GZ, and WT, but growth promotion in dry weight was only recorded for cultivars BI, EE, and WT (Figure 14).

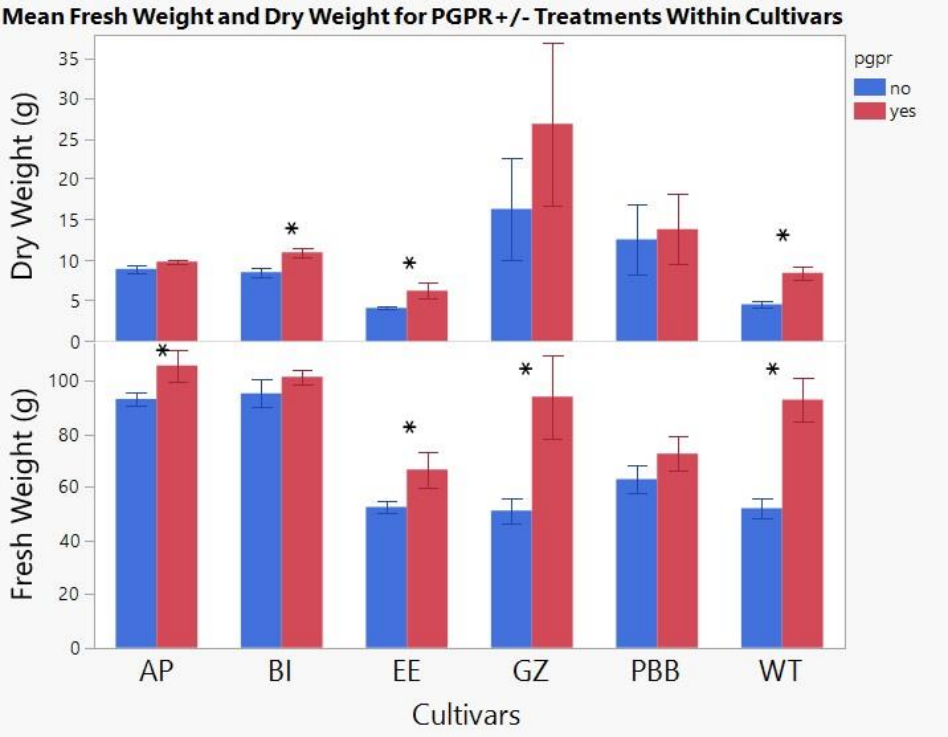


Figure 14. Average fresh weight and dry weight of both PGPR+ and PGPR- heirloom tomato plants by cultivar at 6 weeks. Error bars indicate standard error. Asterisk indicates significant difference ($p<0.05$).

3.2 Microbial Analysis

In terms of the microbial association with the fruit surface washes for the heirloom cultivars, we recorded a statistically lower level of *Salmonella* across all of the heirloom varieties compared to the modern varieties ($p< 0.001$) (Figure 15).

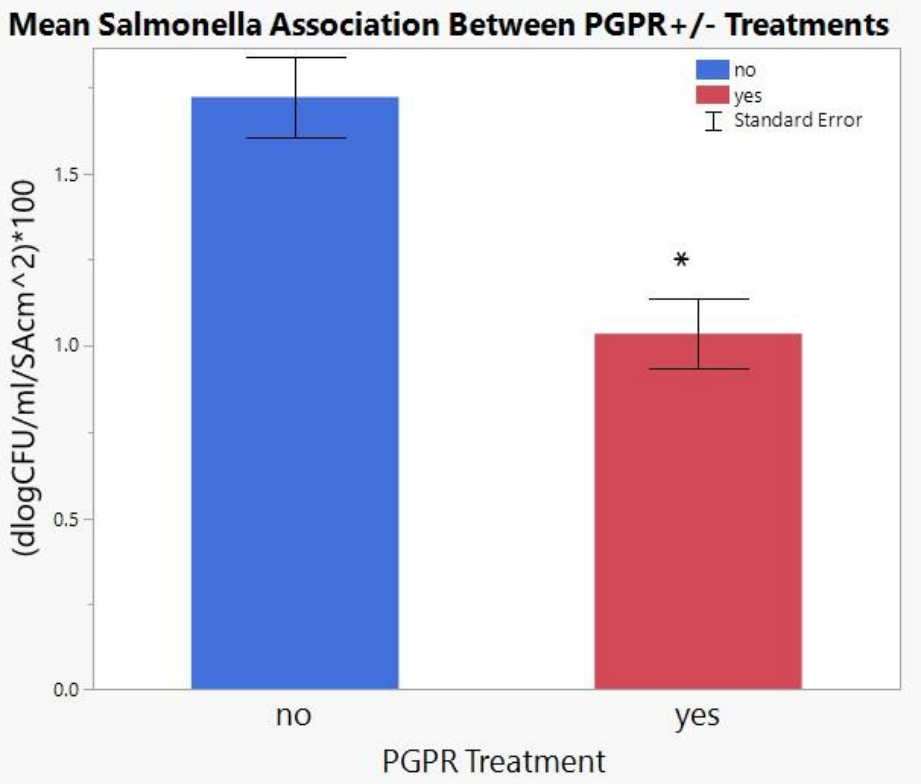


Figure 15. Analysis of *Salmonella* growth on exudate washes of Heirloom varieties with and without PGPR. Error bars indicates stnd error. Asterisk indicates significant differences. ($p<0.05$)

Breaking down the data by individual cultivar, I analyzed the impact of PGPR inoculation on *Salmonella* outcomes, in a one way t-test,. Cultivars BI and WT, demonstrated lower *Salmonella* association in the treated group compared to the non-inoculated group ($p<0.05$). Additionally, cultivars Ap ($p=0.08$), EE ($p=0.1$) and GZ ($p=0.1$) approached significantly lower *Salmonella* growth in the tretaed group compared to the untreated group (Figure 16)

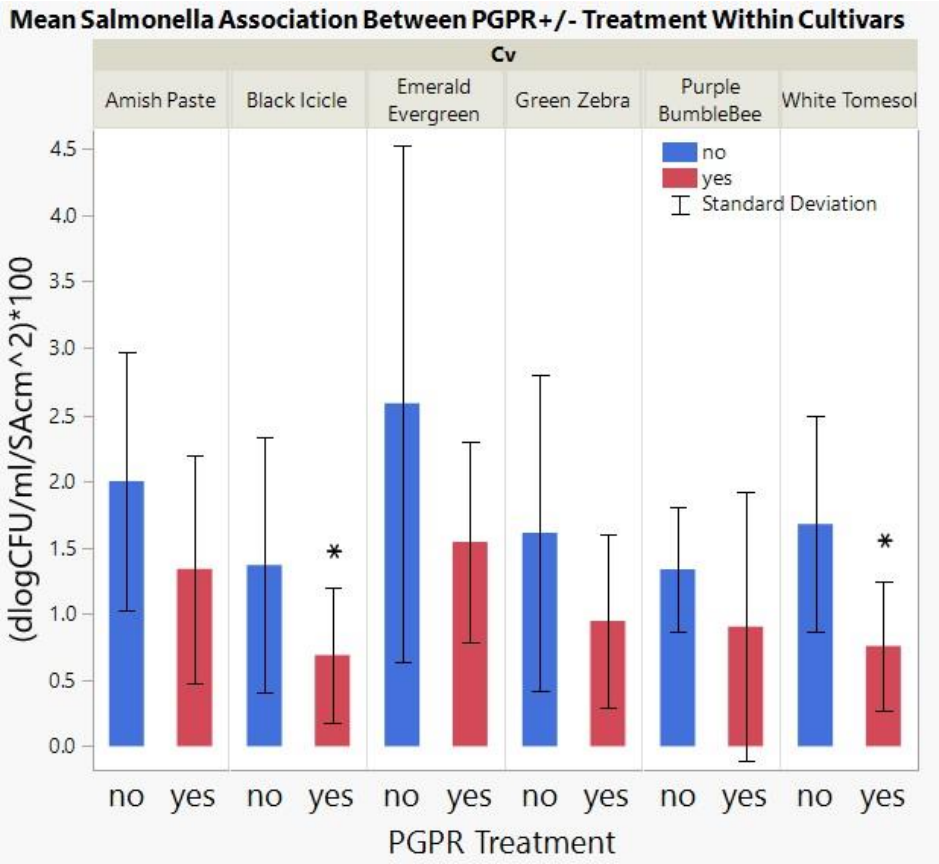


Figure 16. Analysis of *Salmonella* growth on exudate washes within Heirloom varieties PGPR+/-. Error bars indicate stnd error. Asterisks indicate significant differences. (p</=0.05)

3.3 Sugar Analysis

Similarly to the previous chapter, we investigated the phytochemical tomato fruit surface changes caused by the PGPR inoculation starting with the total sugar quantification across all cultivars. While the treated group had lower levels of total sugars in the fruit surface washes, it

was not a statistically significant difference (*p*=0.119), however removing three outliers (GZ and WT/PGPR- and EE/PGPR+) gave a *p*-value of 0.06 (data not shown). (Figure 17).

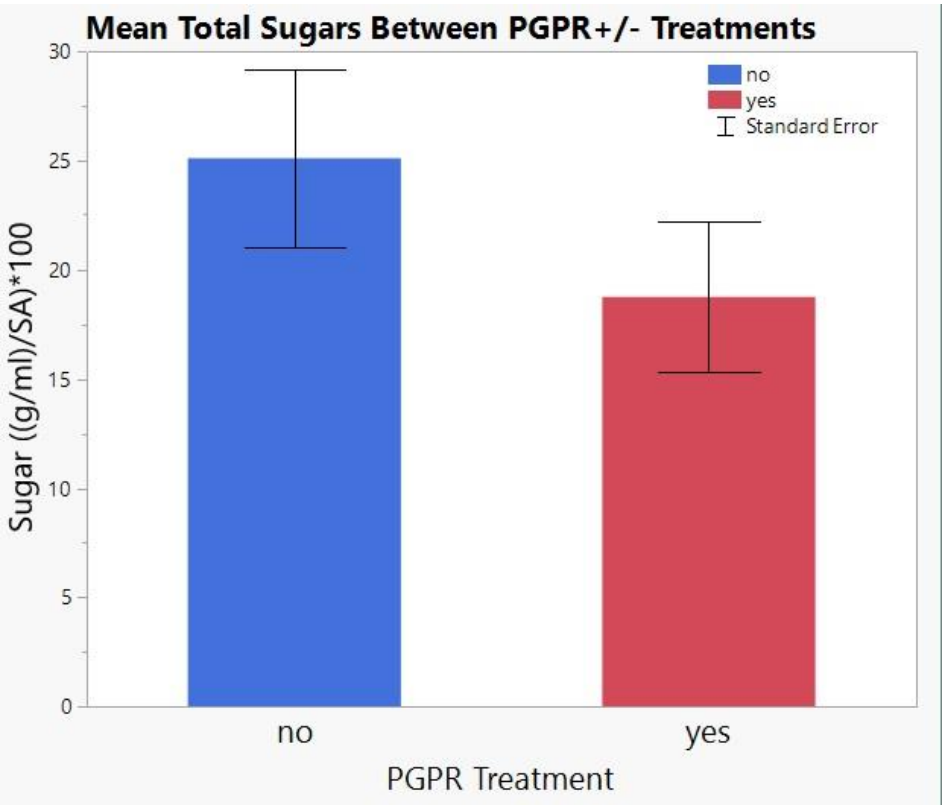


Figure 17. Analysis of Sugar analysis within Heirloom varieties for both PGPR +/- . Error bars indicate stnd error. Asterisk indicates significant differences. ($p<0.05$)

Assessing the impacts of PGPR treatment by cultivar, the cultivars BI (*p*<0.01) had lower total sugars in the treated group compared to the untreated group. No other cultivar showed any significant differences (Figure 18).

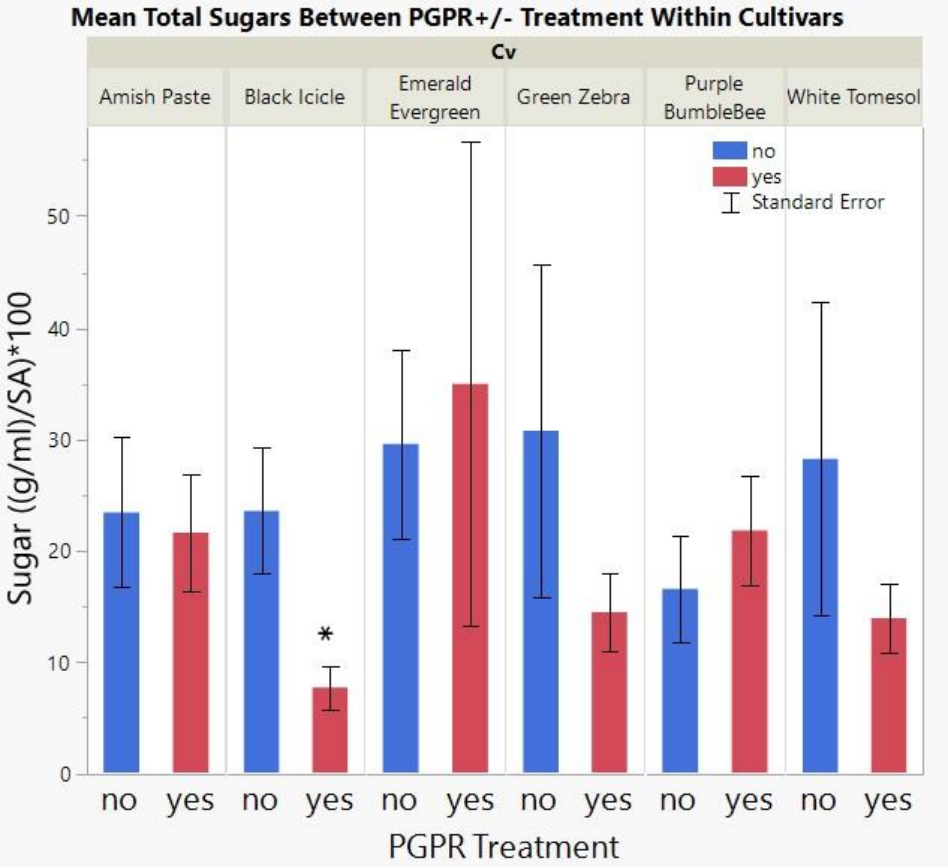


Figure 18. Analysis of Sugar analysis within Heirloom varieties for both PGPR +/-. Error bars indicate stnd error. Asterisks indicate significant differences. ($p<0.05$)

3.4 Citric Acid Analysis

Another comparison was made between the PGPR+/- treated groups based on the content of citric acid in the fruit surface washes. We observed a significant difference in the citric acid

levels where the group treated with PGPR had a lower level of citric acid compared to the untreated group ($p<0.001$) (Figure 19).

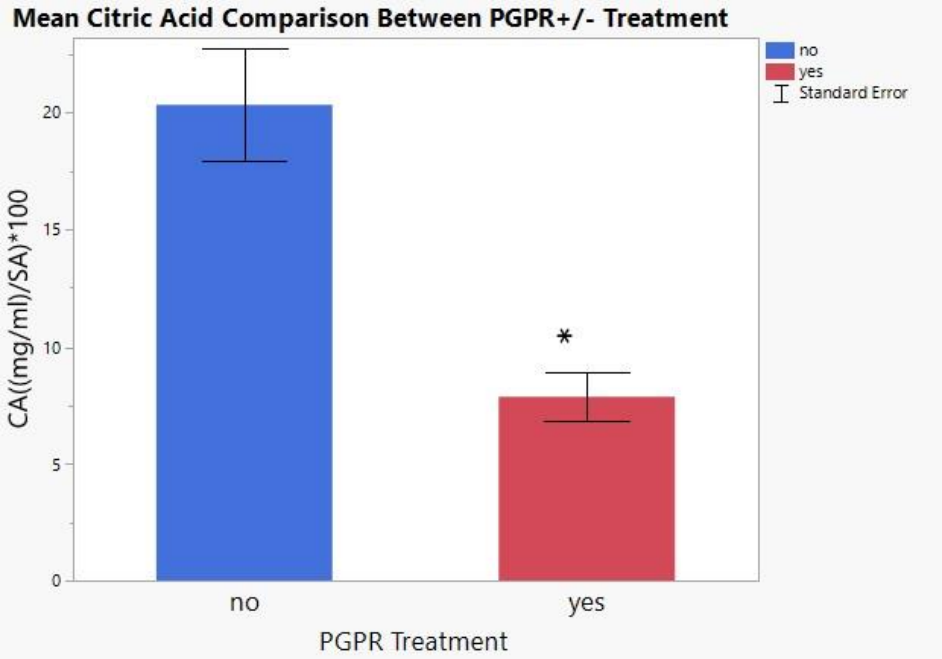


Figure 19. Average citric acid of fruit surface washes for PGPR+ and PGPR- treated heirloom tomato plants. Error bars indicate standard error. Asterisk indicates significant difference ($p<0.05$).

Breaking down data by individual cultivars, every heirloom cultivar, other than cultivar PBB, demonstrated significantly lower citric acid levels in the treated group compared to the untreated group ($p<0.05$) (Figure 20).

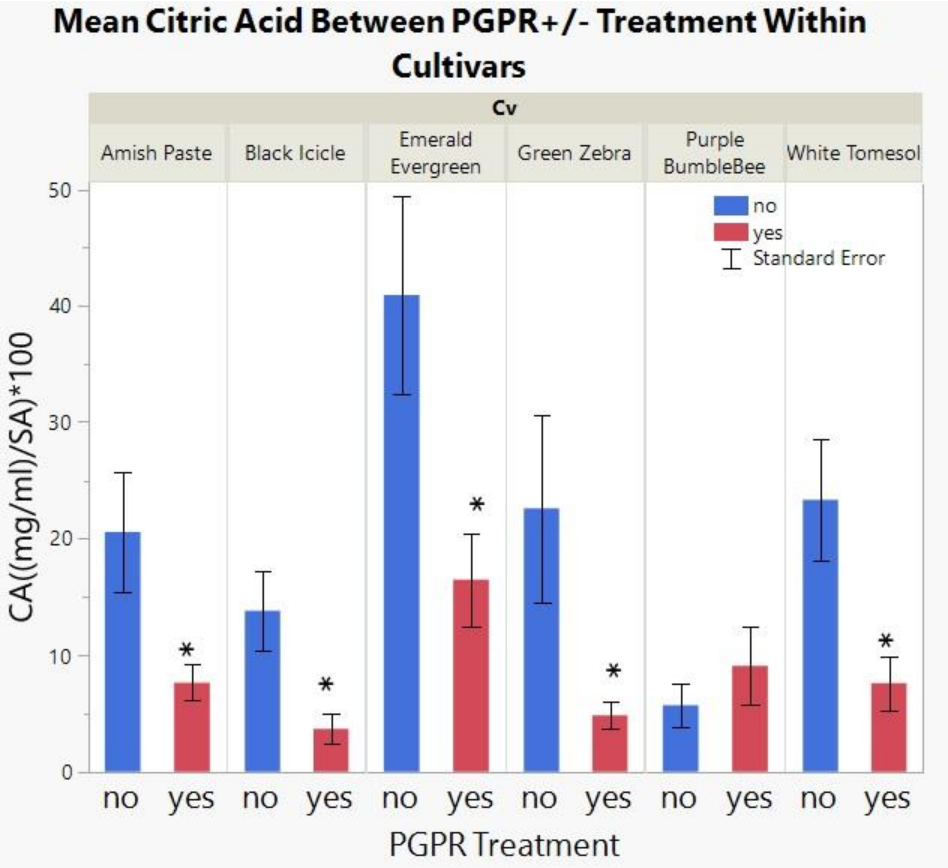


Figure 20. Average citric acid of fruit surface washes for PGPR+ and PGPR- treated heirloom tomato plants across cultivars. Error bars indicate standard error. Asterisk indicates significant difference ($p<0.05$).

3.5 Fatty Acid Analysis

The largest comparison in this research project was comparing the fatty acid levels between the PGPR+/- groups. We first looked at the fatty acid levels of each peak identified (myristic acid,

hexadecanoic acid, linoleic acid, linolenic acid, stearic acid, erucic acid, and arachidic acid) in the GCMS spectra across all heirloom cultivars. We observed that the levels of the unsaturated fatty acids linoleic acid and linolenic acid (both $p<0.05$), and the saturated fatty acids myristic ($p<0.05$), stearic ($p=0.06$) and palmitic ($p=0.078$) acids were all higher in the treated group compared to the untreated group. All other fatty acid levels showed no significant differences across all heirloom cultivars (Figure 21).

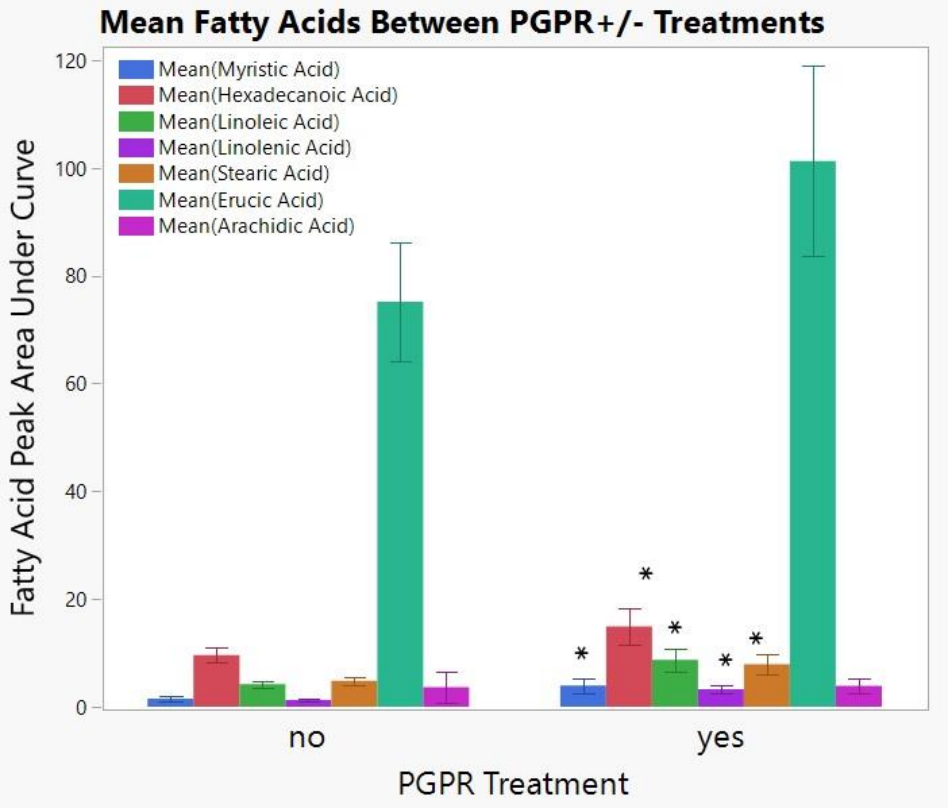


Figure 21. Average fatty acids of fruit surface washes for PGPR+ and PGPR- treated heirloom tomato plants. Error bars indicate stnd error. Asterisks indicate significant difference (p</=0.078).

For the saturated fatty acids, cultivar AP showed higher myristic (p=0.07) acids in the PGPR treated group, and cultivar EE showed higher hexadecanoic acid, stearic acid, and arachidic acid (all p<0.01) in the PGPR treated group. All other saturated fatty acids for the other cultivars exhibted no significant differences (Figure 22).

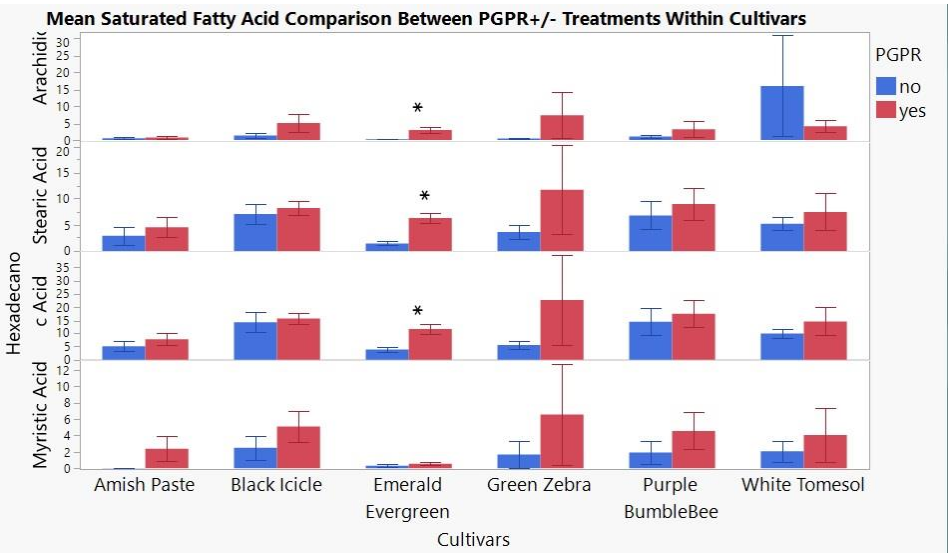


Figure 22. Average saturated fatty acids of fruit surface washes for PGPR+ and PGPR- treated heirloom tomato plants across cultivars. Error bars indicate stnd error. Asterisks indicates significant difference (p<0.05).

In the unsaturated fatty acids group, cultivar EE had higher levels of linoleic acid (p<0.01) in the treated group while cultivar PBB had higher levels of linolenic acid (p<0.05) in the treated group compared to the untreated group. Cultivars AP, EE (both (p=0.08) and BI (p=0.098) showed a weak significant difference for linolenic acid in which the treated group had a higher level compared to the untreated group. All other cultivars had no significant differences between the unsaturated fatty acid levels (Figure 22).

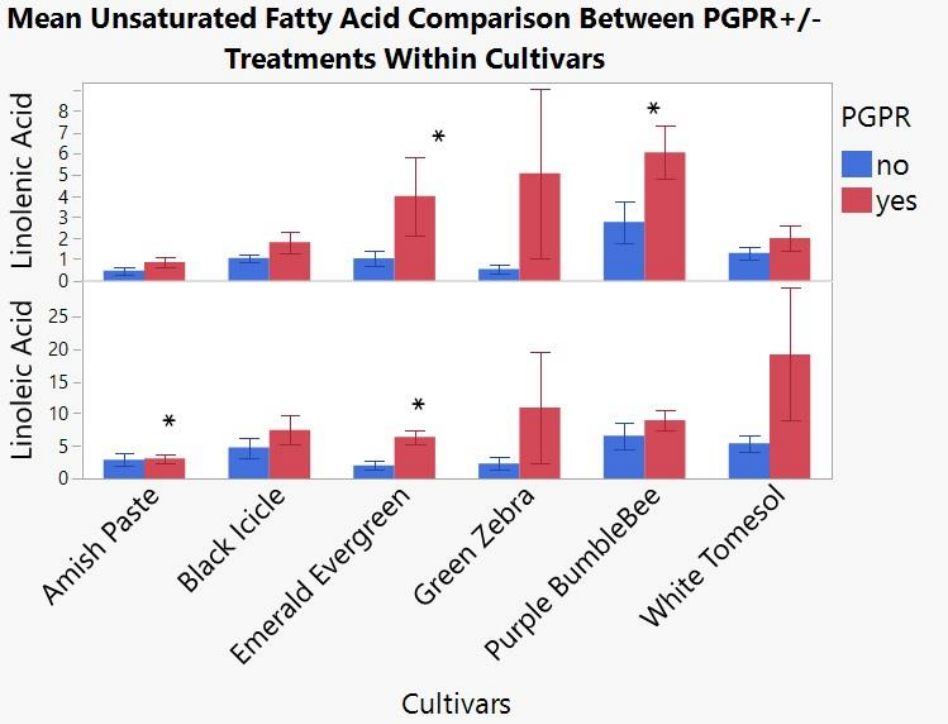


Figure 23. Average unsaturated fatty acids of fruit surface washes for PGPR+ and PGPR- treated heirloom tomato plants across cultivars. Error bars indicate stnd error. Asterisks indicates significant difference (p</=0.08).

Finally, we investigated the monounsaturated fatty acid, erucic acid, in which only cultivar EE demonstrated higher levels of erucic acid (p<0.01) in the treated group compared to the untreated group. All other cultivars failed to show any significant difference between the treated and untreated groups (Figure 23).

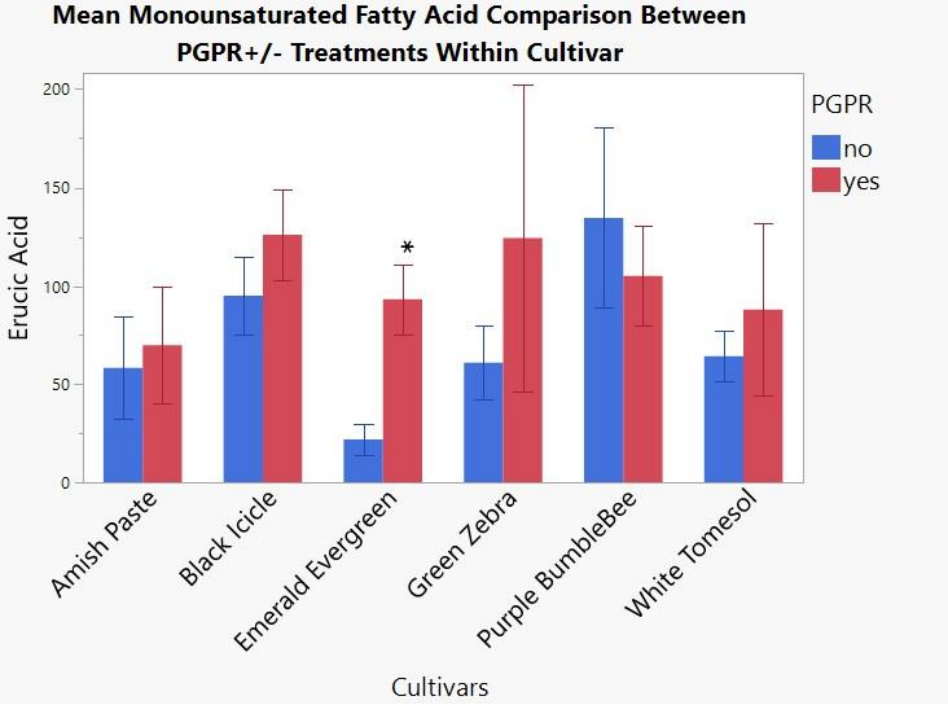


Figure 24. Average monounsaturated fatty acid levels of fruit surface washes for PGPR+ and PGPR- treated heirloom tomato plants across cultivars. Error bars indicate stnd error. Asterisks indicates significant difference (p<0.05).

3.6 Relationship between phytochemicals in fruit washes and *Salmonella* growth

Employing a regression model with the phytochemical metabolites to investigate the variation in *Salmonella* growth, we included sugars, citric acids, and fatty acids for both PGPR+/- groups. For the PGPR- group, the regression model explained 34% of the *Salmonella* variation significantly ($R^2=0.34$, $p<0.01$) with citric acid, sugars, and linolenic acid ($p<0.05$) being positively correlated with microbial growth, and hexadecanoic acid being negatively correlated with microbial growth ($p<0.05$). Meanwhile for the PGPR+ regression model, the model could explain 17% of the *Salmonella* variation weakly significantly ($R^2=0.17$, $p=0.08$) with stearic acid being positively correlated with microbial growth ($p<0.05$) and both myristic acid and hexadecanoic acid being negatively correlated with microbial growth ($p<0.05$) (Table 2 Appendix).

3.7 Color and Texture Analysis

The final component of this investigation was a comparison between the PGPR treated and untreated groups in regards to the tomato fruit color and texture values. First, we looked at color scale values across all cultivars, we found no significant differences in Hue, Chroma, or Lightness between the PGPR+/- groups. (Figure 25)

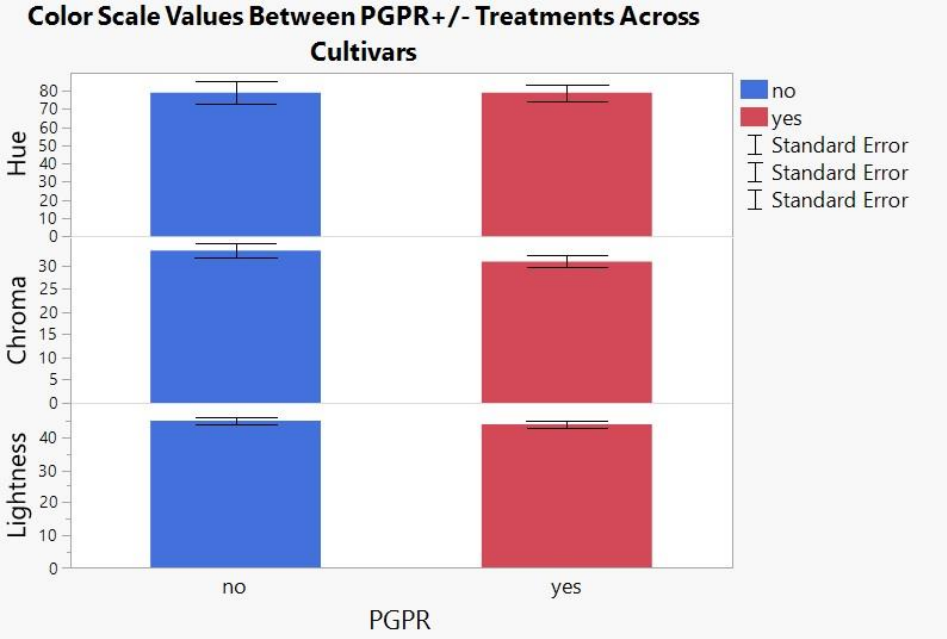


Figure 25. Analysis of color scale values between PGPR+/-. Error bars indicate stnd error. Asterisk indicates significant differences. ($p<0.05$)

Breaking down the data by cultivar, "Lightness" was significantly lower in the PGPR treated groups for cultivars GZ and PBB. Color value "Chroma" was lower for PGPR treated groups for cultivars BI, GZ, and PBB, and value "Hue" was higher in cultivar EE. ($p<0.05$) (Figure 26).

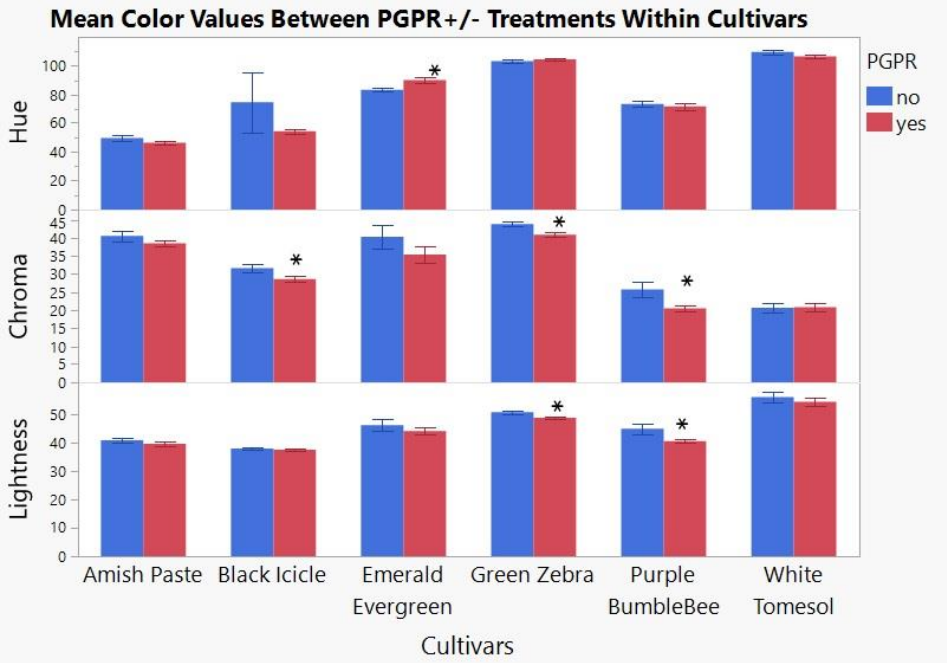


Figure 26. Analysis of color scale values between PGPR+/- groups within cultivars. Error bars indicate stnd error. Asterisks indicate significant differences. ($p<0.05$).

We were able to detect higher stiffness values in the PGPR treated groups across all cultivars compared to the untreated groups ($p<0.05$) (Figure 27).

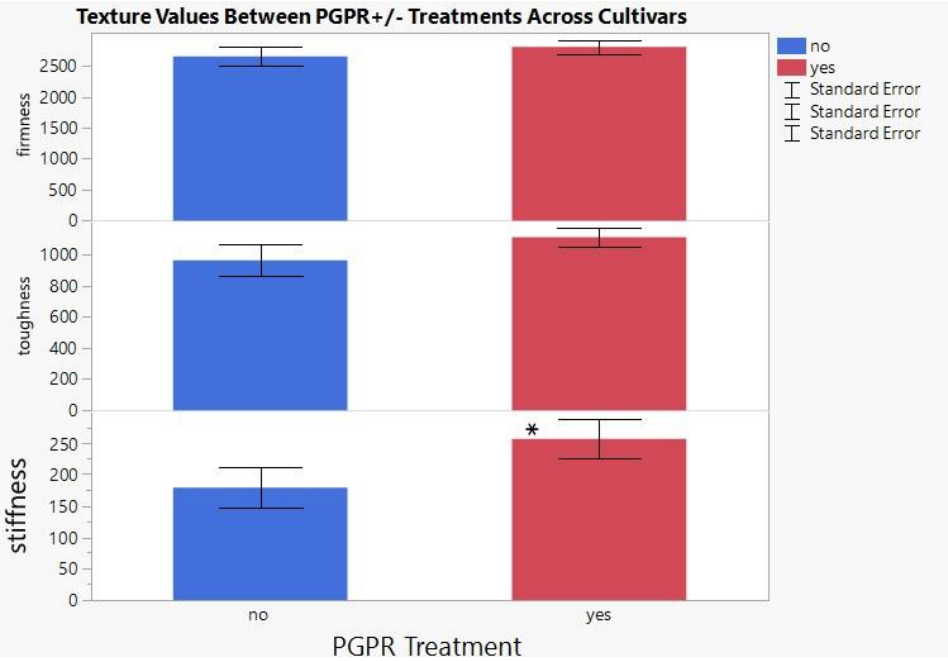


Figure 27. Analysis of texture values between PGPR+/- groups. Error bars indicate stnd error. Asterisks indicate significant differences. ($p<0.05$).

Lastly, we compared the differences in texture values between the PGPR treated and untreated groups for each of the cultivars. We measured weakly significant higher stiffness ($p=0.056$) and toughness ($p=0.086$) values in cultivar AP. We also measured higher stiffness ($p=0.088$) in the treated group of cultivar EE. All other cultivars did not demonstrate significant differences between the treated and untreated groups ($p<0.05$) (Figure 28).

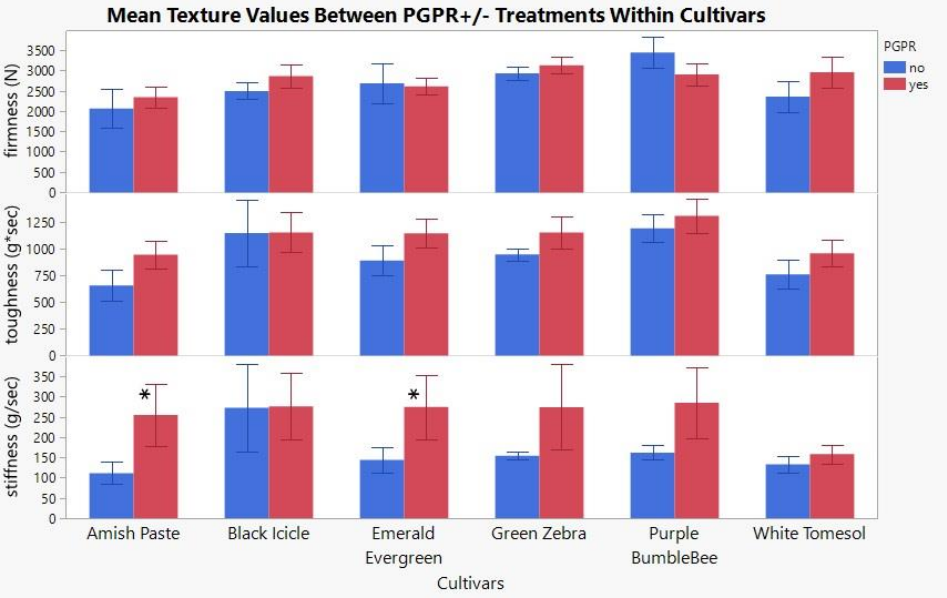


Figure 28. Analysis of texture values between PGPR+/- groups for each cultivar. Error bars indicate stnd error. Asterisk indicate significant differences. ($p<0.05$).

3.8 Relationship between color/texture values in fruit washes and *Salmonella* growth

Employing a regression model with the phytochemical metabolites to investigate the variation in *Salmonella* growth, we included both color and texture values for both PGPR+/- groups. For the PGPR- group, the regression model explained 18% of the *Salmonella* variation, but was not significant ($R^2=0.18$, $p=0.62$). Meanwhile for the PGPR+ regression model, the model could explain 55% of variation significantly ($R^2=0.55$, $p<0.01$) with Hue being positively correlated with microbial growth ($p<0.05$), Lightness being negatively correlated with microbial growth

($p<0.05$), and firmness being weakly negatively correlated with microbial growth ($p=0.052$) (Table 3 Appendix).

4. Discussion and Conclusion

4.1 Salmonella Association Discussion

As previously described in the literature review, the inoculation of PGPR, specifically *Pseudomonas* rhizobacteria, have been known to elicit a multitude of positive changes in agricultural plants including abiotic and biotic stress tolerance. In this chapter, we investigated the impact of *Pseudomonas* on modulating heirloom tomato fruit surface phytochemical profiles and the resulting connection to *Salmonella* association. Firstly, in, we determined that across all cultivars the treated group showed lower *Salmonella* association, and we also saw that all cultivars other than PBB demonstrated at least weakly significantly lower microbial association. With the knowledge of what cultivars and to what degree the inoculation of these tomato plants with PGPR occurred, we can address other variables that the PGPR inoculation had on the tomato plants and the properties on the tomato fruit surfaces that could help explain these differences.

4.2 PGPR Growth Promotion, Color Texture Discussion

We then wanted to measure if there was an actual impact on the plant growth to the six different cultivars in order to determine if there was indeed an induced response from the plants and subsequent plant growth. Interestingly, we saw plant growth promotion in the PGPR treated group for either or both fresh and dry weights for every cultivar except PBB. Most cultivars showed fresh weight growth, and cultivar EE showed the most fresh weight and dry weight

growth. Cultivar BI was the only one that showed only dry weight growth promotion and not fresh weight growth promotion. It is possible that there were issues fully drying the plant weights in the ovens, leading to inconsistent data there. Overall, though it depends on the cultivar, we did see growth promotion from the PGPR for most plants. Another component to consider is how the PGPR impacted the fruit surface color and texture qualities. Across all cultivars, the PGPR treatment did not alter color scale values, but it did result in higher stiffness. Within the cultivars, we saw that cultivars PBB and GZ with the PGPR treatment had lower Lightness and Chroma levels. Additionally, cultivar BI showed lower Chroma levels while cultivar EE demonstrated higher Hue levels in the PGPR treated group. Within cultivars for the texture values, only cultivars AP and EE demonstrated higher stiffness values in the PGPR treated group. Furthermore, regression analysis only showed significance for the PGPR+ group where Hue was positively correlated with *Salmonella* growth and Lightness and firmness were negatively correlated with microbial growth. This data suggests the inoculation of tomato plants with PGPR *Pseudomonas* could potentially modulate pigmentation metabolites (Marvasi et al; 2014) such as anthocyanins and carotenoid levels such as lycopene (Zameer et al; 2016), which could explain the lower lightness and chroma levels in some of the PGPR inoculated cultivar, and the higher hue level in the cultivar EE inoculated group. This could also be related to reduced *Salmonella* association as anthocyanins and lycopene have demonstrated anti-microbial properties (Hraishawi et al; 2020, Ma et al; 2019). Only one previous study has investigated the use of PGPR on the firmness of tomato fruits and they also observed increased firmness in the inoculated group compared to the non-inoculated group and suggested that ethylene modulation from the PGPR impacting ethylene pathways in the plant could hypothetically be the cause (Mena-Violante & Olalde-Portugal, 2007).

4.3 Role of Sugars and Citric Acid in *Salmonella*-tomato interaction

Some of the phytochemical metabolites modulated by the inoculation of PGPR studied were total sugars and citric acid,. While across all cultivars, total sugars were not significantly decreased by the PGPR, we do see that at least for cultivar BI (which was also less impacted in plant growth promotion), that total sugars were reduced, which could partially explain the decrease in *Salmonella* association as a means of reducing available nutrients for continued persistence. Although our study looked at total sugars, *Salmonella* is known to have a preference for glucose compared to other sugars present. It is possible that different cultivars exuded a higher level of glucose compared to other sugars in the fruit surfaces washes, which could hypothetically explain the differences in correlations. Meanwhile for citric acid, we observed decreased citric acid in the surface washes across all cultivars except for cultivar PBB (which was not impacted in terms of plant growth). This aligns with the impact of PGPR on plant growth and citric acid reduction. Regression analysis for PGPR+/- groups showed positive correlation between sugars/citric acid and microbial growth only in PGPR- group. Citric acid at high levels have shown anti-microbial properties for *Salmonella* (Jeanne-Marie et al; 1997) (Al-Nabulsi at al; 2014). It is possible that at lower levels *Salmonella* could be utilizing citric acid that gets converted to citrate to metabolize as a nutrient source, explaining the positive correlation (Wynosky-Dolfi at al; 2014). The lack of correlation and lower levels of citric acid in the PGPR inoculated group could be a result of metabolic changes from the PGPR, but this is contrary to previous findings of increased citric acid in tomato fruits with PGPR inoculation (González Rodríguez et al; 2018) (Kalozoumis et al; 2021). It is possible that our work with

different PGPR strains and investigations with heirloom tomato varieties, as well as focusing just on the tomato surface contributed to different findings.

4.4 Fatty Acids Discussion

Fatty acids in previous studies were seen to be correlated with negative *Salmonella* growth for tomato fruits. For our PGPR analysis across all cultivars, we found that the unsaturated linoleic and linolenic acids, as well as the saturated myristic acid, hexadecanoic acid, and stearic acid were all in weakly significant higher accumulation for the PGPR treated group compared to the untreated group. Looking at the individual cultivars, cultivar EE demonstrated the highest accumulation of fatty acids, while cultivar AP had an increase in stearic acid, and PBB had an increase in linolenic acid. Cultivar EE demonstrated the highest *Salmonella* association in PGPR uninoculated plants and a significant reduction in *Salmonella* favorability when plants were inoculated with PGPR. This cultivar also had the greatest changes in fatty acid profiles, suggesting that the lower the initial fatty acid levels were more strongly induced by PGPR and may have contributed to reducing microbial association on the tomato fruit surface. Regression analysis demonstrated a positive correlation for linolenic acid with microbial counts and a negative correlation with hexadecanoic acid with microbial counts in the non-PGPR group. For the PGPR-inoculated group, regression analysis showed stearic acid being positively correlated with microbial growth and both myristic and hexadecanoic acids being negatively correlated with microbial growth. Once again, we see that the impact of PGPR differs between cultivars. In some of these cultivars, and perhaps in even some of the individual sample batches of tomato fruits, there was more of an increase in certain fatty acid accumulations that resulted in reduced *Salmonella* growth. Unfortunately sample sizes were too small to do regression analysis on each

individual cultivar for both PGPR+/- groups. Within both PGPR treated and untreated groups, hexadecanoic acid was negatively associated with *Salmonella* growth. For the PGPR-treated group, it is possible the inoculation of PGPR induced an increase in myristic acid that provided an additional inhibitory effect to *Salmonella* and that the PGPR induced a large enough increase in hexadecanoic acid to counter the supportive effect of the stearic acid induced increase.

4.5 Conclusion

Across all of our studies, it appears the impact of PGPR *Pseudomonas* inoculation in tomato plants on their fruit surface depends on the cultivar and the phytochemical metabolite. For example, PGPR didn't have an impact on fresh weight growth promotion for cultivar BI, but the application of PGPR did reduce sugar levels and *Salmonella* association. This means that compared to other cultivars, BI may have increased dark-colored anthocyanin content (resulting in reduced Chroma) and reduced the release of sugars into the surface wash from the application of PGPR, thus limiting *Salmonella* growth. However, the application of PGPR impacting plant growth promotion successfully for the cultivars also aligned with reduction in citric acid levels for the treated cultivars, where all except cultivar PBB had both impacted by PGPR treatment. Meanwhile, other cultivars like EE demonstrated increased plant growth promotion as well as most of the fatty acids increased in accumulation, suggesting a method of increased antagonistic metabolites to limit *Salmonella* association. The induced systemic responses from PGPR on tomato plants are not entirely known, and with the variety in heirloom varieties in their size, coloration, and inherent phytochemical profile differences, it appears that PGPR impacts these different cultivars in different ways. Some of these alterations could by limiting metabolites that

Salmonella can synthesize and use as nutrients to persist, or by means of increasing metabolites that would impede upon *Salmonella* synthesis of nutrients, thus limiting their persistence on the fruit surface washes. Much of the variation from the regression analysis could not be explained from the metabolites and color/texture values investigated here, meaning that much of the *Salmonella* association seems to be from other metabolites than what was investigated here. Complicating our data analysis further is that much of the regression analysis could not be run within the cultivars due to small sample sizes. Future investigations would do well to focus on anti-microbial metabolites that could be increased from PGPR such as carotenoids, anthocyanins and other VOCs that could limit *Salmonella* growth, and to focus on the direct genetic impacts of PGPR on these synthesis pathways. Additionally, greater success with PGPR in reducing *Salmonella* could be achieved in using different species of PGPR, or even a co-inoculum of multiple species for greater symbiosis.

Chapter 5: Conclusions and Future Directions

The purpose of this research was divided into two main sections. In chapter 3, we investigated the correlations between the phytochemical profiles of the fruit surfaces of heirloom and modern tomato varieties as well as some of the impacts of fruit surface color and texture qualities. We determined that the heirloom varieties supported a lower level of *Salmonella* compared to the modern varieties ($p=0.06$), yet the heirloom varieties contained larger levels of sugars and citric acids that were themselves correlated with higher *Salmonella* suggesting that other metabolites or factors may play a more important role in the differences in microbial association. Some of the variation within the heirloom varieties proved puzzling such as the cultivar EE which had similar sugar levels as other cultivars but was far more favorable to *Salmonella*. Some of this variation can be explained by differences in citric acid levels, fatty acids, and to some degree differences in coloration levels that differed from other cultivars such as BI and PBB that were less favorable to *Salmonella*. Another factor between heirloom and modern cultivars could be that the heirloom varieties may have had higher or lower levels of anthocyanins or terpenoids present compared to the modern varieties, but these metabolites were not measured in this research. While the modern varieties were grown in large flow tunnels, the heirloom varieties were often crowded together in small tables in the greenhouse, potentially resulting in volatile compounds from the glandular trichomes rubbing off on the heirloom fruit surface, which could in part explain some of the differences in *Salmonella* growth as well. Another factor could be that the heirloom varieties (being typically smaller in surface area compared to the modern varieties) could have had higher levels of stiffness and firmness, resulting in lesser *Salmonella* association, but coloration and texture data was not measured for the modern cultivars. Finally, fatty acids were not measured in the modern varieties, and it is possible that certain fatty acids

were higher in heirloom cultivars, but again these were not measured for modern varieties. Future studies when looking at the phytochemical profiles of heirloom and modern varieties would do well to analyze some of these blind spots we encountered in this study. In chapter 4, we analyzed the impact PGPR *Pseudomonas* had on modulating the phytochemical profile of various tomato fruits as well as the impact on color and texture quality, and the resulting impacts of *Salmonella* association. Similarly to the non-PGPR analysis in chapter 3, we found a large degree of variation in the heirloom cultivars. Furthermore, we found a trend where the cultivars that demonstrated more increased plant growth promotion from the PGPR treatment also demonstrated some of the more exaggerated alterations in metabolite accumulation. We found total sugars to be weakly positively correlated with *Salmonella* growth, and we also found citric acid to be positively correlated with *Salmonella* growth. Meanwhile, we found that some fatty acids (specifically hexadecanoic acid in both treatment groups and myristic acid in the PGPR-treated group) were associated with inhibited *Salmonella* growth. The inoculation of PGPR resulted in a small degree of sugar reduction and a reduction in citric acid as well as slight increases in certain fatty acids (depending on the cultivar), reinforcing the idea that not only are sugars and citric acid beneficial to *Salmonella* growth while some fatty acids are inhibitory, but that PGPR can induce a systemic response in tomato fruits that limit favorable metabolites for *Salmonella* while increasing unfavorable metabolites. Additionally, the PGPR treatment appeared to increase the stiffness of fruit surfaces, which could result in higher fatty acids accumulating in the tomato fruit surfaces and reducing available sugars and citric acids. Oddly enough, the inoculation of PGPR appeared to impact different cultivars in different ways. While most cultivars demonstrated increased plant growth, cultivar PBB (the only cherry variety) did not demonstrate different plant growth promotion. The fatty acid levels of this cultivar also did

not change from the inoculation, but this cultivar also had some of the highest fatty acid levels without the inoculation. Meanwhile cultivar EE, which had some of the lowest fatty acid levels without the PGPR inoculation demonstrated some of the most universally dramatic increases in fatty acids. It is possible that there may be a threshold in which how much metabolites can be altered in the tomato fruit surfaces when inoculated with PGPR. Some of these changes could also be increasing pigment molecules that could be inhibitory to *Salmonella*. Future studies would do well to investigate more clearly some of the metabolites not measured in modern cultivars and to go further to investigate some of the potential inhibitory impacts of terpenoids, volatile compounds, and anthocyanins both presumably present to varying degrees in diverse heirloom varieties and modern varieties. Connecting the impact of PGPR on these specific metabolites for both heirloom and modern varieties may be able to help explain more of the variation seen in *Salmonella* growth. What could also improve some of the PGPR results seen in this study could be to do a co-inoculum with other microbe or fungal PGPR varieties, or to try several different individual strains to see if certain symbiotic relationships would work better than others. These results ultimately shed light on some of the complex relationships between enteric human pathogens, plant hosts, human health, and symbiotic microbes, and are the first to include and focus on heirloom tomato varieties. While more research is needed, this work helps pave the way for potential genetic breeding programs, and organic means of introducing biofertilizer rhizobacteria into agricultural practices to modulate the phytochemical profiles of tomato fruits to reduce *Salmonella* association at a pre-harvest stage, ultimately reducing foodborne illness outbreaks, improving the state of agriculture, and helping to build a better world.

Appendix 1: Supplementary tables

Table 1. Regression analysis using sugars and citric acid as variables to explain *Salmonella* variation between modern and heirloom cultivars.

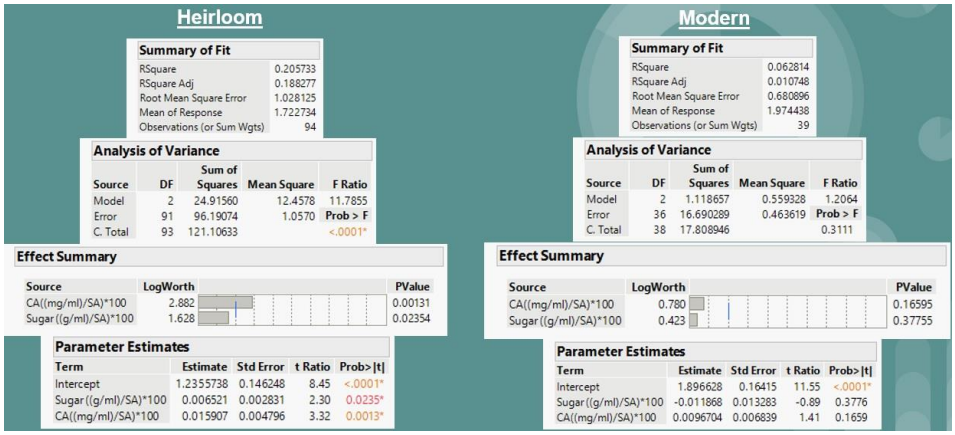


Table 2. Regression analysis using sugars, citric acid, and fatty acids as variables to explain *Salmonella* variation between PGPR+/- groups.

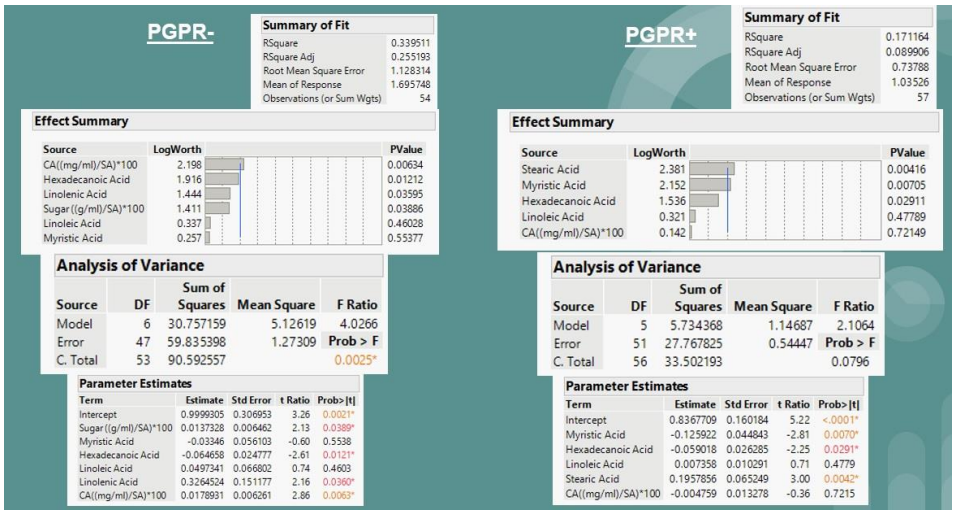
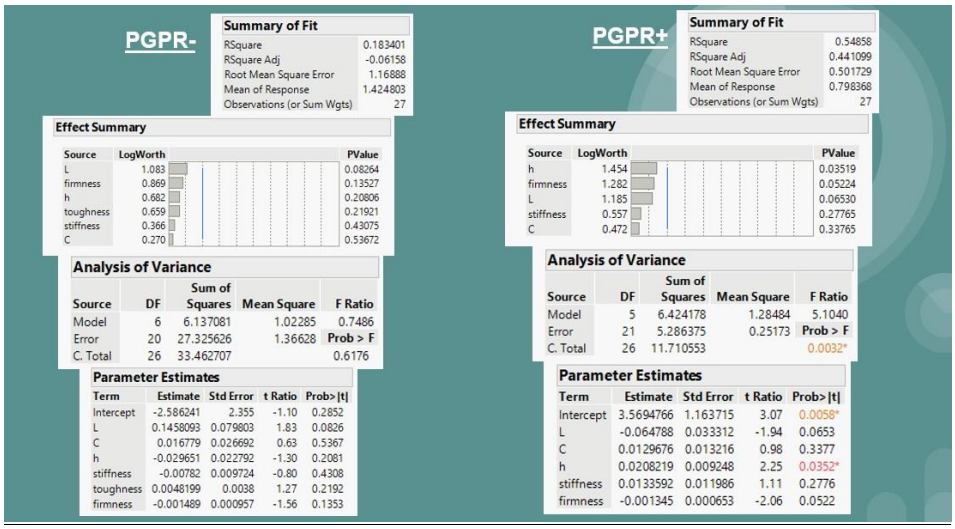


Table 3. Regression analysis using color and texture values as variables to explain *Salmonella* variation between PGPR+/- groups.



PGPR-

Summary of Fit

RSquare	0.183401
RSquare Adj	-0.06158
Root Mean Square Error	1.16888
Mean of Response	1.424803
Observations (or Sum Wgts)	27

Effect Summary

Source	LogWorth	PValue
L	1.083	0.08264
firmness	0.869	0.13527
h	0.682	0.20806
toughness	0.659	0.21921
stiffness	0.366	0.43075
C	0.270	0.53672

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	6	6.137081	1.02285	0.7486
Error	20	27.325626	1.36628	Prob > F
C. Total	26	33.462707		0.6176

Parameter Estimates

Term	Estimate	Std Error	t Ratio	Prob>|t|
Intercept	-2.586241	2.355	-1.10	0.2852
L	0.1458093	0.079803	1.83	0.0826
C	0.016779	0.026692	0.63	0.5367
h	-0.029651	0.022792	-1.30	0.2081
stiffness	-0.00782	0.009724	-0.80	0.4308
toughness	0.0048199	0.0038	1.27	0.2192
firmness	-0.001489	0.000957	-1.56	0.1353

PGPR+

Summary of Fit

RSquare	0.54858
RSquare Adj	0.441099
Root Mean Square Error	0.501729
Mean of Response	0.798368
Observations (or Sum Wgts)	27

Effect Summary

Source	LogWorth	PValue
h	1.454	0.03519
firmness	1.282	0.05224
L	1.185	0.06530
stiffness	0.557	0.27765
C	0.472	0.33765

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	5	6.424178	1.28484	5.1040
Error	21	5.286375	0.25173	Prob > F
C. Total	26	11.710553		0.0032*

Parameter Estimates

Term	Estimate	Std Error	t Ratio	Prob>|t|
Intercept	3.5694766	1.163715	3.07	0.0058*
L	-0.064788	0.033312	-1.94	0.0653
C	0.0129676	0.013216	0.98	0.3377
h	0.0208219	0.009248	2.25	0.0352*
stiffness	0.0133592	0.011986	1.11	0.2776
firmness	-0.001345	0.000653	-2.06	0.0522

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