ABSTRACT

Title of Dissertation: BACTERIAL COMMUNITIES OF THE SPECIALTY CROP PHYLLOSPHERE: RESPONSE TO BIOLOGICAL SOIL AMENDMENT USE, RAINFALL, AND INSECT VISITATION

Sarah Michelle Allard, Ph.D., 2016

Dissertation directed by: Dr. Shirley Ann Micallef, Assistant Professor, Department of Plant Science and Landscape Architecture

Microorganisms in the plant rhizosphere, the zone under the influence of roots, and phyllosphere, the aboveground plant habitat, exert a strong influence on plant growth, health, and protection. Tomatoes and cucumbers are important players in produce safety, and the microbial life on their surfaces may contribute to their fitness as hosts for foodborne pathogens such as *Salmonella enterica* and *Listeria monocytogenes*. External factors such as agricultural inputs and environmental conditions likely also play a major role. However, the relative contributions of the various factors at play concerning the plant surface microbiome remain obscure, although this knowledge could be applied to crop protection from plant and human pathogens. Recent advances in genomic technology have made investigations into the diversity and structure of microbial communities possible in many systems and at multiple scales. Using Illumina sequencing to profile particular regions of the 16S rRNA gene, this study investigates the influences of climate and crop management practices on the field-grown tomato and cucumber microbiome. The first research chapter (Chapter 3) involved application of 4 different

soil amendments to a tomato field and profiling of harvest-time phyllosphere and rhizosphere microbial communities. Factors such as water activity, soil texture, and field location influenced microbial community structure more than soil amendment use, indicating that field conditions may exert more influence on the tomato microbiome than certain agricultural inputs. In Chapter 4, the impact of rain on tomato and cucumber-associated microbial community structures was evaluated. Shifts in bacterial community composition and structure were recorded immediately following rain events, an effect which was partially reversed after 4 days and was strongest on cucumber fruit surfaces. Chapter 5 focused on the contribution of insect visitors to the tomato microbiota, finding that insects introduced diverse bacterial taxa to the blossom and green tomato fruit microbiome. This study advances our understanding of the factors that influence the microbiomes of tomato and cucumber. Farms are complex environments, and untangling the interactions between farming practices, the environment, and microbial diversity will help us develop a comprehensive understanding of how microbial life, including foodborne pathogens, may be influenced by agricultural conditions.

BACTERIAL COMMUNITIES OF THE SPECIALTY CROP PHYLLOSPHERE: RESPONSE TO BIOLOGICAL SOIL AMENDMENT USE, RAINFALL, AND INSECT VISITATION

by

Sarah Michelle Allard

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Advisory Committee:
Dr. Shirley Micallef, Chair
Dr. Christopher Walsh
Dr. Andrea Ottesen
Dr. Stephanie Yarwood
Dr. Amy Sapkota

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Chapter 1: Introduction

Plants support diverse microbial populations both above- and below-ground, and these communities are intimately connected to plant health and function (Bulgarelli et al., 2013; Philippot et al., 2013; Pieterse et al., 2014; Vorholt, 2012). While some microbes that colonize plants may be classified as plant or human pathogens, other symbionts may promote plant growth or stimulate response to stress or infection, qualities that could be managed and capitalized upon in agricultural systems (Bakker et al., 2012; Berg, 2009; Berlec, 2012; Lakshmanan et al., 2014). Agricultural inputs such as manure-derived fertilizers or environmental factors like rainfall could potentially lead to abiotic and biotic changes influencing microbial dynamics in the plant habitat. Disruption of plant-associated microbial community structures could, in turn, lead to changes in plant vitality and susceptibility to enteric and plant pathogenic microorganisms. Due to the intimate association between plants and their microbial symbionts, an assessment of the influence of these inputs on microbial community structure and diversity will lead to a more comprehensive view of plant health and function.

Of increasing concern in the agricultural environment is the presence of foodborne pathogens, such as *Salmonella enterica*, pathogenic *Escherichia coli*, and *Listeria monocytogenes* (Brandl, 2006; Olaimat and Holley, 2012). Unlike plant pathogens, which often decrease crop yield and quality (Oerke, 2006), these are unlikely to cause visible symptoms while the crop is growing, making detection in the field difficult. Enteric pathogens are capable of survival through harvest and processing (Bennett et al., 2015;

Harris et al., 2003; Tomás-Callejas et al., 2011), and once established on the plant surface they are difficult to eliminate (Goodburn and Wallace, 2013; Parish et al., 2003), posing a serious threat to consumers of fresh-cut produce. In the United States alone, the CDC estimates that 1 in 6 people (48 million total) become sick from foodborne illness every year, and 3,000 of these illnesses result in death (Scallan et al., 2011a; Scallan et al., 2011b). Of these illnesses, 46% are estimated to be produce-associated (Painter et al., 2013). Efforts to reduce foodborne disease contamination of fresh produce today primarily focus on prevention, both pre- and post-harvest. Good Agricultural Practices (GAPs) have been established to give farmers recommendations on cultural practices that strengthen on-farm food safety risk management by reducing opportunities for contamination through wildlife management, effective waste processing, worker education and training, and microbiological water quality management (FDA et al., 1998). Although the plant-foodborne pathogen interaction is an important factor in understanding on-farm food safety, a holistic approach that includes agricultural and environmental conditions and the plant microbiome as contributors to this interaction will lead to a more complete understanding of how to reduce the risk of foodborne pathogen contamination of produce.

Without knowing what organisms are present in the phyllosphere and how environmental conditions influence the communities there, research cannot move forward to providing direct answers that will help farmers in prevention efforts. There is a clear need for replicated manipulative studies in the field of phyllosphere microbiology, and with next generation sequencing becoming more accessible and affordable, it is now feasible to

characterize the complexity of microbial communities in the phyllosphere and to investigate influential factors. The application of these methods in the area of food safety of fresh produce is still in early stages, and there are many basic questions that need to be answered for the field to move forward. Based on the hypothesis that environmental and human-driven factors in agriculture may influence the crop microbiome in a way relevant to food safety and security, the purpose of this project was to investigate response of bacterial community structure and diversity associated with tomato (roots, flowers, leaves, and fruit) and cucumber (fruit) to three factors: application of organic and synthetic fertilizers, rainfall prior to harvest, and insect exclusion.

The objectives and specific aims of the study were as follows:

Objective 1: Evaluate the influence of three soil amendments (raw poultry litter, sterile poultry litter pellets and vermicompost) on the tomato microbiome as compared to mineral nutrition.

> Specific aim 1-1: Compare community composition and structure among blossom, fruit, and root surfaces from plants fertilized with a variety of organic amendments versus a synthetic fertilizer.
>
> Specific aim 1-2: Evaluate physiochemical soil characteristics and their relationship to bacterial community composition and structure on tomato plant surfaces.
>
> Specific aim 1-3: Determine if relative abundance of taxa of interest for food safety, such as Enterobacteriaceae, Listeriaceae, Clostridiaceae and Paenibacillaceae are statistically significantly different among treatments.

Objective 2: Assess the influence of rain events on bacterial community structure of field-grown fresh produce.

Specific aim 2-1: Profile and compare bacterial communities living on the surface of cucumber fruits, tomato leaves, and tomato fruits 4 days before, 1 day after, and 4 days after rain events.

Specific aim 2-2: Determine if relative abundance of taxa of interest for food safety, such as Enterobacteriaceae, Listeriaceae, Clostridiaceae and Paenibacillaceae shifts over the three time points.

Objective 3: Evaluate the influence of insect exclusion on bacterial communities inhabiting the surfaces of tomato blossoms and fruit**.**

Specific aim 3-1: Compare bacterial community structure and diversity between tomato fruit and blossoms collected from netted plants and non-netted plants.

Specific aim 3-2: Identify taxa that are likely introduced or augmented by insect visitation to tomato blossoms and fruit.

Chapter 2: Literature Review

1. **The plant microbiome: Diversity, dynamics, and role in agriculture**

Microorganisms, including bacteria, fungi, oomycetes, and viruses, contribute to many processes important to human health and economic wellbeing, including global nutrient cycling, plant and animal health, and the phytoremediation of toxic chemicals (Ali et al., 2012; Furnkranz et al., 2008). Increasingly, studies focused on human medical conditions include a microbial component, as researchers turn toward the microbiome to explain differences in human health outcomes (Clemente et al., 2012; Ley et al., 2005; Turnbaugh et al., 2007). Microbes make up a substantial portion of our own biomass and play an important role in human disease incidence, obesity, and behavior (Flint et al., 2012; Sampson and Mazmanian, 2015; Turnbaugh et al., 2009). Similarly, plants support numerous and diverse microbial communities that are intimately connected to their health and function (Berendsen et al., 2012; Bulgarelli et al., 2013; Lakshmanan et al., 2014; Lindow and Brandl, 2003; Philippot et al., 2013; Vorholt, 2012). The large collective genome of microbes associated with plants is often termed the plant's "second genome" (Berendsen et al., 2012) due to the importance of microbes in plant growth and response to stress.

In regards to crop production, plant-associated microbial communities influence yield, quality, safety, and disease management. Study of the agricultural plant microbiome is therefore relevant to consumers, farmers, and health officials due to its influence on human health, economic stability, and food safety. Recent advances in molecular technology have changed the state of plant microbiome research, allowing for replicated

high-throughput studies under manipulated conditions. These advances have allowed scientists to characterize microbiota and interpret functional importance for diverse habitats. This literature review examines current and classic research on the plant microbiome, with a focus on microbial dynamics of the phyllosphere in relation to agriculture.

2. The phyllosphere and rhizosphere

The interactions between plants and their associated microbes are complex and varied. One scientist coined the phrase, "Microbes wear their guts on the outside" (Janzen, 1985) to emphasize the importance of microbes in plant growth and defense, and the intimate relationship that exists between the two. Research into the community structure and function of epiphytic bacteria, fungi, and archaea associated with plants is most often divided into two plant regions. The phyllosphere constitutes the aboveground surfaces of the plant, mostly composed of the leaves but also including blossoms, fruit, and stems, while "rhizosphere" describes the area immediately surrounding plant roots (Vorholt, 2012). Differences in nutrient availability and environmental pressures between the two plant regions contribute to their distinct microbial diversity and community distribution. While unique, both the phyllosphere and the rhizosphere support complex microbial communities (Bodenhausen et al., 2013; Ottesen et al., 2013). Bacteria are the most numerous inhabitants of the phyllosphere and rhizosphere (Bulgarelli et al., 2013; Vorholt, 2012), although filamentous fungi and yeasts are also present (Andrews and Harris, 2000; Inácio et al., 2002). Bacterial volume is estimated at up to 10^{11} cells per gram in the rhizosphere and up to 10^{8} cells per gram in the phyllosphere (Berendsen et al., 2012; Lindow and Brandl, 2003).

The phyllosphere is an ephemeral environment compared to the rhizosphere; seasonal changes in foliage or differences in annual versus perennial lifestyle can lead to drastic habitat changes for microbiota in the phyllosphere (Vorholt, 2012). While rhizosphere microbiota may survive in the absence of roots, adjusting to survive in the fairly constant conditions of the soil, phyllosphere microorganisms would be left without a comparable secondary habitat if, for example, a host plant died or dropped its leaves in the winter months. The phyllosphere is a harsh environment, characterized by wild fluctuations in nutrients, water availability, and temperature, as well as constant exposure to damaging ultraviolet radiation and plant-derived reactive oxygen species (Lindow and Brandl, 2003; Muller and Ruppel, 2014; Vorholt, 2012). Despite these challenging conditions, many microorganisms have adapted to survive in the phyllosphere, on the leaf surface, in the leaf apoplast, or on the surface of flowers and fruit (Rastogi et al., 2013). The phyllosphere provides an enormous surface area for microbial colonization, comprising one of the largest microbial habitats on earth (Bulgarelli et al., 2013; Vorholt, 2012).

3. Drivers of microbial community structure

Microbial life associated with plants differs across multiple scales, from landscape to plant genotype to plant organ microsite. Many studies have sought to establish a hierarchy of importance for potential factors influencing bacterial community structure in the phyllosphere and rhizosphere, however results have differed across different host plants, locations, and environmental factors.

3.1 Plant genotype

Plant genotype has a documented influence on microbial community structure in multiple systems (Micallef et al., 2009a; van Overbeek and van Elsas, 2008; Whipps et al., 2008). In the rhizosphere, root exudates and root morphology differ across plant species and cultivars, shaping the structure and function of rhizosphere microbial communities in conjunction with soil type (Berg and Smalla, 2009). Root exudates, including nutrients and secondary metabolites, may enhance or decrease survival of certain microbial constituents of the rhizosphere. Rhizodeposits used as carbon sources, including sugars, mucilage, root border cells, and dead root cap cells, vary in composition and volume depending on plant genotype and environment (Jones et al., 2009), as do antimicrobial and growth-promoting secondary metabolites (Wink, 2003). From the pool of highly diverse microorganisms in the soil environment, plants may recruit certain organisms suited to the rhizosphere and beneficial for plant growth and protection, allowing these organisms to increase in abundance and activity (Berendsen et al., 2012; Kim et al., 2011; Micallef et al., 2009b; Philippot et al., 2013). This recruitment extends to the phyllosphere as well, where differences in plant exudates drive microbial activity and community structure. Even at the cultivar level, leaf and fruit exudate profiles may differ, causing differential success for specific bacteria (Han and Micallef, 2014; Han and Micallef, 2016). Geographic location has been identified as a driving factor in many studies profiling phyllosphere communities (Belisle et al., 2012; Finkel et al., 2011; Perazzolli et al., 2014; Rastogi et al., 2012), however other studies have found limited influence of geographic location on phyllosphere communities of plants with the same genotype (Redford et al., 2010). Studies have shown that different plant species growing

close together in the same soil harbor diverse phyllosphere microbial communities (Delmotte et al., 2009; Knief et al., 2012), indicating that plant genotype may be a stronger driver of microbial community structure than geographic location.

3.2 Plant organ

Within a specific plant, microbial abundance and diversity differ between the phyllosphere and rhizosphere in general, but differences are clear at even finer scales. On tomato plants, microbial diversity has been shown to decrease as distance from the soil increases, and surface dwelling microbial communities differ between lower, upper, and middle leaves, as well as between blossoms, fruits, and roots (Ottesen et al., 2013). While abundance and diversity of phyllosphere microbiota may vary based on factors including environmental conditions and plant genotype, several dominant phyla, including Proteobacteria, Actinobacteria, Bacteriodetes, and Firmicutes, are generally consistent on the leaf surface (Bulgarelli et al., 2013; Rastogi et al., 2013; Whipps et al., 2008). Flower-associated microbial communities also may support a fairly consistent core microbiome, which contains several of the same bacterial taxa as well as additional fungal constituents (Aleklett et al., 2014). Fruit and vegetable surfaces support common taxa as well, but diversity and community structure vary by plant type; tree fruits such as apples and peaches share similar community structure, as do tomatoes and peppers (Leff and Fierer, 2013). At the family level, many produce types support a high abundance of Enterobacteriaceae, often in conjunction with a relatively low species richness (Leff and Fierer, 2013). This has relevance for food safety, as several foodborne pathogens commonly associated with fresh produce outbreaks are classified as Enterobacteriaceae, a family apparently well adapted to the phyllosphere.

3.3 Plant microsite

At even finer spatial levels, such as between the trichomes and stomata of leaves, or between the stigma and style of flowers, microbial communities may be distinct, with some microbes being preferentially attracted to certain exudates and characteristics specific to these microsites (Aleklett et al., 2014; Leveau and Lindow, 2001; Miller et al., 2001). In fact, individual microbial species may use different strategies to survive on diverse microsite habitats. Transcriptional studies have shown that *Pseudomonas syringae,* a common constituent of the phyllosphere, exhibits motility on the leaf surface but not in the apoplast, instead expressing genes that enhance resistance to plant defense response in the apoplast (Yu et al., 2013). Similarly, *Methylobacterium extorquens* expression differs between epiphytic leaves, roots, and synthetic growing medium, producing more abundant proteins related to stress response and alternative substrate utilization in the phyllosphere (Gourion et al., 2006). In flowers, yeast species differ significantly between inner and outer corolla, as well as between floral rewards (nectar and pollen) and the rest of the flower structure (Pozo et al., 2012). Microbial structure and function appears to be highly specialized to microsites within all plant organs, at even the smallest scale.

3.4 Season and development

Microbial communities can be dynamic throughout the seasons or the life cycle of plant tissues, adding a temporal component to the study of the plant microbiome. Apple flowers host a diverse array of microbes, with community changes occurring consistently over time from before flower opening through flower senescence (Shade et al., 2013). Similarly, spinach leaves have shown that leaf-surface dwelling microbial communities

become more complex and change in structure throughout the life of the leaves (Lopez-Velasco et al., 2013). In the rhizosphere of *Arabidopsis thaliana*, differences in abundance of certain phyla were observed between several developmental time points (Chaparro et al., 2014; Micallef et al., 2009a). Other studies, however, have found that leaf surface-dwelling microbes are fairly consistent over the development of the leaf (Delmotte et al., 2009). These studies indicate that the microenvironments present on plant surfaces are variable enough to support significantly different communities on various temporal, developmental, and spatial scales. However, the relative importance of these factors in shaping microbial community structure and diversity remains unclear. Clarification of the spatiotemporal dynamics of phyllosphere communities could help elucidate the when, where, and why of pathogen infection in plants.

3.5 Environment and agricultural management

In conjunction with the plant habitat over different spatial and temporal scales, different environmental factors and agricultural management practices may contribute to bacterial community structure in the phyllosphere. Rainfall, for example, has been linked to changes in the prevalence of non-pathogenic indicators of fecal contamination in lettuce fields (Xu et al., 2016) and also larger scale bacterial community changes (Copeland et al., 2015). The use of organic versus conventional management, encompassing a variety of differences in pest and nutrient management approaches, may lead to the maintenance of distinct phyllosphere (Ottesen et al., 2009) and rhizosphere bacterial communities (Bulluck et al., 2002; Esperschuetz et al., 2007). Studies investigating the individual components of these management strategies have produced varied results. Biological and chemical pesticide application appears to have little influence on phyllosphere microbial

community structure in grape systems (Perazzolli et al., 2014), while copper pesticide application had a weak but significant effect on tomato phytobiomes (Ottesen et al., 2015). Biological soil amendments, used in organic agriculture as sources of plant nutrition, have shown some influence on rhizosphere communities (Das and Dhar, 2012; Esperschuetz et al., 2007; Jangid et al., 2008; Lavecchia et al., 2015; Peiffer et al., 2013), however in some cases the influence of soil was inconsistent and unclear (Gao et al., 2015; Tatti et al., 2012; Tian and Gao, 2014). In the phyllosphere, the influence of soil amendment application and many other agricultural management practices remain to be investigated.

4. Microbial adaptation to the phyllosphere

4.1 Establishment

Inoculum for initial colonization of the phyllosphere may come from a variety of sources including air, water, seed, soil, or animal vectors (Aizenberg-Gershtein et al., 2013; Bulgarelli et al., 2013; Lopez-Velasco et al., 2013; Rastogi et al., 2012; Ushio et al., 2015; Vorholt, 2012). Within plant genotypes, specific organs of the phyllosphere have demonstrated core communities, with the same, often plant species- and organ-specific, phyla appearing year to year despite aging or newly established hosts (Knief et al., 2010). This indicates one or more environmental reservoirs of complex microbial inoculum as well as plant characteristics that support the establishment of certain microbes on a consistent basis (Vorholt, 2012). For most plants, soil and phyllosphere communities fail to share many common constituents, exhibiting clear differences in dominant phyla (Bodenhausen et al., 2013; Kim et al., 2012; Knief et al., 2012). Air, another potential source of inoculum, may be an important factor in early phytobiome establishment, but

its influence may be specific to certain developmental time points or plant species (Fahlgren et al., 2010; Maignien et al., 2014; Vokou et al., 2012). The consistency of plant microbiome communities across seasons and generations could be partially explained by transmission from other nearby plants serving as microbial reservoirs (Vorholt, 2012). Vertical transmission from seed to plant represents another potential avenue of inoculum, sometimes termed the "maternal effect." Lopez-Velasco et al. found that some core phyla, including Proteobacteria, Firmicutes, and Acidobacteria, are present both in seeds and at multiple stages of spinach plant development. The number of distinct OTUs nearly tripled between the cotyledon and full-grown plant stages, however, indicating that any maternal effect was only a part of a greater picture, necessitating additional sources of inoculum (Lopez-Velasco et al., 2013). Microbial inoculum may travel between plants through pollinators and other animal visitors, however more research is need to clarify these relationships (Aleklett et al., 2014; Pozo et al., 2012; Ushio et al., 2015). How these different sources of inoculum work together with the plant to craft the microbiome remains unclear.

4.2 Adhesion and motility

The vast majority of microbes in the phyllosphere are commensal and do not cause an immune reaction in the plant. Most phyllosphere bacteria are non-motile and lack flagellin, a commonly detected molecular associated molecular pattern (MAMP) that can induce plant immune responses. One extensively studied and common phyllosphere microorganism, *Pseudomonas syringae*, is motile and, indeed, is recognized by plants. Instead of adapting to the epiphytic leaf environment by using limited available nutrients, *P. syringae* moves to find nutrients through chemotaxis (Yu et al., 2013). In general,

phyllosphere bacteria practice adhesion over motility, often forming multi-species biofilms for protection from rainfall, desiccation, osmotic stress, UV radiation, and other harsh environmental conditions (Morris et al., 1998; Rastogi et al., 2013). These aggregates, made of extracellular polysaccharides, also allow for quorum sensing, which contributes to epiphytic growth and success (Carlier et al., 2015). *Pseudomonas syringae* maintains both motility and biofilm-forming abilities in the phyllosphere. Studies have shown that aggregate formation in the phyllosphere leads to enhanced survival compared to a solitary lifestyle (Monier and Lindow, 2003).

4.3 Survival

Once established in the phyllosphere, microorganisms have several tools at their disposal to enhance survival and enable reproduction. Most exhibit oligotrophic (slow-growing) characteristics, which help them adapt to the inconsistent and sparse conditions of the phyllosphere (Vorholt, 2012). Some of the most prevalent bacterial colonizers of the phyllosphere utilize compounds commonly emitted from leaves to support metabolism and enhance protection. Bacteria uptake various forms of nitrogen using amino acid transporters (Delmotte et al., 2009). Carbon appears to be more limiting than nitrogen in the phyllosphere (Wilson and Lindow, 1994), and microbial communities have diverse strategies for acquiring enough carbon. *Methylobacterium* has been identified as part of the core microbiota of several plant species (Delmotte et al., 2009), and it produces methylotrophic enzymes that allow the bacteria to use plant-released methanol for metabolism (Knief et al., 2012). Microbes unable to metabolize methanol may adopt another strategy by releasing indole-3-acetic acid, which loosens plant cell walls and stimulates the release of saccharides (Fry, 1989; Lindow and Brandl, 2003). Some

bacteria in the phyllosphere produce energy from light to supplement their metabolism. Light harvesting bacteriorhodopsins, previously described in exclusively aquatic systems, have been identified on the surfaces of leaves from several plant genera (Atamna-Ismaeel et al., 2012a; Atamna-Ismaeel et al., 2012b) and are a phylogenetically diverse and abundant component of the phyllosphere (Atamna-Ismaeel et al., 2012a).

Aggregate formation and exopolysaccharide release help protect microbes from desiccation, but water scarcity also contributes to osmotic stress in the phyllosphere. Osmoprotectants, small compounds such as choline and trehalose that balance osmotic difference between a cell and its surroundings, may be produced by microbes themselves or derived from plants (Chen and Beattie, 2008; Freeman et al., 2010). These osmoprotectants enhance survival of epiphytic bacteria in the phyllosphere.

Oxygen from plant photosynthesis as well as light exposure can lead to reactive oxygen species (ROS)-related damage to microbial cellular components including nucleic acids, proteins, and lipids (Vorholt, 2012). Pigmentation is one strategy by which microorganisms limit exposure and avoid this type of damage. Some of the most successful colonizers of the phyllosphere, *Methylobacterium*, *Sphingomonas*, and *Pseudomonas*, are pigmented (Lindow and Brandl, 2003). One study showed that *Clavibacter michiganensis* mutants deficient in pigmentation exhibit reduced survival and abundance in the peanut phyllosphere, while other phyllosphere inhabitants showed a range of susceptibilities to UV radiation (Jacobs et al., 2005). DNA repair systems, including release of photolyase enzymes help microbes recover from UV-associated

damage (Vorholt, 2012). Microbes colonizing flowers are somewhat protected from both UV exposure and desiccation due to the structure of the flower (Aleklett et al., 2014), but they may risk exposure to antimicrobials released by the flower (Pozo et al., 2012).

Phyllosphere microbiota, though usually commensal, may stimulate immune responses from the plant or release of antimicrobial compounds from other nearby microorganisms. Both leaves and blossoms release a variety of secondary metabolites with antimicrobial properties (Pozo et al., 2012; Wink, 2003), necessitating some form of adaptation of phyllosphere microbiota to withstand exposure. Some fungi, including *Trichoderma*, produce hydrophobins, hydrophobic proteins that coat their own cell surfaces and protect their hyphal tips from degradation by plant-released compounds. Others produce proteins that bind to cellulose, enhancing attachment and enabling modification of the plant cell wall (Hermosa et al., 2012).

5. Constituents of the plant microbiome

5.1 Commensal plant-microbe associations

Symbiotic relationships such as mutualism, parasitism, and commensalism drive the dynamic between plants and their associated microbial communities. The vast majority of microbes in the phyllosphere and rhizosphere are commensal, exerting no negative influence on plant growth and development and, in fact, often confer a positive effect (Bulgarelli et al., 2013). Many microbes benefit by associating with plants, receiving a habitat for colonization and exudated phytocompounds for metabolism. Plants may benefit from increased growth and enhanced response to biotic and abiotic stress factors.

In the rhizosphere, nitrogen fixation has been well documented (Berg, 2009; Bulgarelli et al., 2013; Chaparro et al., 2012; Esperschuetz et al., 2007). Rhizobia in the root nodules of legumes and free-living bacteria and archaea fix atmospheric nitrogen to a form usable by plants, receiving carbon exudates and habitat in exchange for providing this service (Philippot et al., 2013). Nitrogen fixation has also been described in the phyllosphere, although not nearly as extensively (Freiberg, 1998; Furnkranz et al., 2008). In the soil, microbes also contribute to enhanced soil stability, which helps increase water retention and uptake. Bacteria, fungi, and yeasts have all shown an ability to release indole-3-acetic acid (IAA), an auxin hormone that can stimulate root growth in plants (Berendsen et al., 2012). By stimulating root growth, these microbes increase the available colonizable surface area in the rhizosphere, potentially allowing for expansion of their own populations. In the rhizosphere, these characteristics have been extensively studied and are now being applied in the field. In the phyllosphere, plant growth promotion has not been investigated in depth and represents an avenue for future research.

Induced systemic resistance has been well characterized in the rhizosphere; rhizosphere microbes stimulate plant defenses, priming them for pathogen attack (Bulgarelli et al., 2013; Haas and Defago, 2005; Shoresh et al., 2010; Yogev et al., 2010; Zamioudis and Pieterse, 2012). Induced systemic resistance has not been consistently described in the phyllosphere, however phyllosphere microbial communities may reduce pathogen colonization by acting as a barrier between the plant surface and the environment. Some commensals may be more adapted to scavenging nutrients from the phyllosphere and may secrete toxic metabolites that reduce pathogen viability. Studies have shown that

axenic (sterile) plants are more susceptible to disease compared to naturally colonized plants (Innerebner et al., 2011). While the mechanisms are not yet well characterized, it is clear that phyllosphere microbiota play a role in reducing pathogen infection in host plants.

5.2 Plant pathogens

Many plant-microbe associations can be beneficial, however pathogenic bacteria and fungi in the phyllosphere and rhizosphere can pose a threat to plant productivity and to food security. While these pathogenic organisms make up only a small part of the phyllosphere and rhizosphere communities, they can have an enormous impact on crop yield. In the United States, approximately 10% of crops are lost to plant pathogens each year, resulting in a multi-billion dollar economic loss (Arora et al., 2012). These pathogens may reduce marketable yield by decreasing growth, distorting crop shape, infecting fruit with toxins, or decreasing shelf life. The dynamics of plant pathogens and the host immune system have been extensively reviewed (Dodds and Rathjen, 2010; Glazebrook, 2005; Jones and Dangl, 2006) and will not be discussed in detail here. However, it is noteworthy that some strategies utilized by plant pathogens may be shared by other microorganisms in the phyllosphere. The use of a type III secretion system (TTSS) appears to be required for many plant pathogens to establish and maintain epiphytic populations in the phyllosphere. This apparatus delivers effector proteins into host cells, suppressing the host defense response if undetected by complementary R proteins, or turning on effector-triggered immunity (ETI) if recognized (Jones and Dangl, 2006). Although TTSS may be required for pathogenesis for many plant pathogens, it can also be used by plant-growth promoting bacteria to enhance survival in the phyllosphere.

Both pathogenic and non-pathogenic growth-promoting bacteria within the genus *Pseudomonas* utilize a type III secretion system apparatus, and some require it for growth (Hirano et al., 1999; Preston et al., 2001).

5.3 Human pathogens

While crop losses due to plant pathogen infection are important to human health in terms of food security, a more direct threat exists in the form of human pathogens, which usually are not pathogenic to the plant itself but can cause serious disease or even death in humans. Human enteric pathogens including *Salmonella enterica* and *Escherichia coli* O157:H7 have been identified as the disease-causing agent in many recent gastroenteritis outbreaks linked to the consumption of fresh produce such as tomatoes, leafy greens, and cucurbits (Greene et al., 2008; Teplitski et al., 2011). Although enteric pathogens are primarily adapted to live within the gut of animal hosts, many of the most virulent microbes have evolved an ability to, at least temporarily, survive on plants (Teplitski et al., 2011; Zheng et al., 2013).

While seasonality may have a stronger effect, certain agricultural management factors have been associated with increased food safety risk (Marine et al., 2015; Pagadala et al., 2015; Strawn et al., 2013). It is becoming increasing clear that certain foodborne pathogens, such as *Salmonella enterica*, may be able to survive long-term in the environment, as observed in surface water and sediment (Bell et al., 2015; Micallef et al., 2012). These enteric pathogens have shown an ability to internalize into plant tissues and may persist if introduced to the environment through contaminated irrigation water or soil (Barak et al., 2011; Guo et al., 2001; Zheng et al., 2013), or through blossom inoculation

(Shi et al., 2009). *Salmonella* colonization of tomato plants is both cultivar-dependent (Barak et al., 2011) and organ-dependent (Zheng et al., 2013). Damage to tomato roots has been shown to increase internalization rates, and certain serovars differentially colonize the flowers, leaves, and roots (Zheng et al., 2013). Type III secretion systems are evolutionarily conserved between plant and animal pathogens (Galan and Collmer, 1999), and studies have shown that TTSS can enhance survival of human pathogens on plants (Barak and Schroeder, 2012).

These pathogens have evolved specific capabilities to adapt to the agricultural environment and can influence phyllosphere and rhizosphere dynamics (Barak and Schroeder, 2012). Flower contamination with enteric pathogens is associated with changes in microbial communities associated with resulting fruits, showing that pathogens can shape phyllosphere community structure (Shi et al., 2009). Adaptation to the plant environment gives these pathogens a competitive advantage, allowing them to use plants as vectors to their next host (Barak and Schroeder, 2012). The *AgfD* gene is a master regulator of aggregative behavior in *Salmonella enterica*, allowing for enhanced leaf attachment (Romling et al., 2000). This gene does not enhance virulence in animal hosts but does enhance survival on plants, showing that the ability of enteric pathogens to survive on plants is not purely due to the presence of conserved mechanisms between plant and animal pathogenicity. The coexistence of human and plant pathogens in the field can support enhanced survival for enteric pathogens. For example, *Xanthomonas perforans*, causal agent of tomato bacterial spot, suppresses PTI (PAMP triggered immunity); when *Salmonella* is present during *X. perforans* infection, survival is

increased tenfold (Potnis et al., 2014). A better understanding of the complex mechanisms by which human enteric pathogens attach to and persist within crop plants may lead to the development of targeted prevention strategies and suggestions to farmers as to how to best prevent foodborne outbreaks originating in their fields.

6. Microbial community characterization

6.1 Culture-independent methods

In light of the impressive influence that microbes have on plant life, it is clear that efforts to improve crop yield, disease resistance, and safety against human pathogens in plants should take into account microbial contributions to these processes. Historically much of the research designed to characterize microbial communities has been dependent upon culturing methods, however culturing selects for only those strains that are able to grow in a lab setting and can vastly underestimate the diversity and abundance of microbes in the environment (Rappe and Giovannoni, 2003). Today, culture-independent methods are becoming more widespread and accessible. Using next generation sequencing technology, it is possible to isolate and sequence DNA and RNA directly from environmental samples, providing a clearer picture of the actual and relative abundance of microbiota in the environment as well as the functional activity of each community. Sequencing-based microbial characterization is a growing field, and it is somewhat unique in the realm of science today in that many studies using these methods are based on data mining rather than hypothesis testing (Wooley et al., 2010). Many studies begin by comparing two or more conditions, looking to see what differs between the two before forming hypotheses and designing follow-up experiments. Advances in technology and in the general body of knowledge of the plant microbiome are making replicated studies

more possible and applicable and hypothesis testing more feasible (Beattie, 2015; Knight et al., 2012).

Sequencing platforms are available from multiple companies; the MiSeq/HiSeq (Illumina, San Diego, CA) and 454 (Roche, Basel, Switzerland) platforms are commonly used today for whole genome and microbial community analysis, but alternative such as PacBio SMRT Sequencing (Pacific Biosciences, Menlo Park, CA) and MinION (Oxford Nanopore Technologies, Oxford, UK), are growing in popularity. Questions to address when characterizing microbial communities include:

1. What is the community structure (identification and abundance of OTUs)?
2. What functions can be carried out (what genes are present)?
3. What functions are actively being carried out (what transcripts and/or proteins are present)?
4. How are community and structure related?
5. How might structure and function change under different treatment conditions?

Different techniques must be utilized to address these questions, and it is important that the method chosen is best suited to the hypothesis being tested.

6.2 Amplicon sequencing (16S/18S/ITS)

Ribosomal RNA may be used to characterize bacterial and fungal communities when identity and diversity of microbes is of primary interest to the study. This is often called amplicon sequencing, as a small region of microbial DNA is amplified from environmental samples using PCR, and sequences are compared to a database or

assembled *de novo* to investigate microbial community structure and diversity (Mizrahi-Man et al., 2013; Muller and Ruppel, 2014). The 16S and 18S regions of the small ribosomal subunit of prokaryotic and eukaryotic organisms, respectively, contain both highly conserved and highly diverse sequences, making them ideal for constructing phylogenies and identifying microorganisms. The Internal Transcribed Spacer (ITS) region may replace 18S in amplicon sequencing of environmental fungi in future studies due to enhanced resolution across a broad range of species (Schoch et al., 2012). Amplicon sequencing may not be the most robust choice if the objective is to investigate functional diversity in microbial communities or activity on a short time scale. DNA may persist in the environment from dormant or dead microorganisms, and live organisms may contribute to function in the community disproportionately to their numerical abundance (Kuczynski et al., 2010). Furthermore, differences in microbial community structure may not necessarily indicate differences in function; resilience and redundancy within communities may allow communities to maintain function under changing conditions (Allison and Martiny, 2008). While this method is far from perfect, it is the most widely used for microbial characterization, and reference databases and data analysis pipelines are currently superior to those for other –omics technologies.

7. Phyllosphere research in agriculture: Future directions

An enhanced understanding of the environmental and human-driven factors that influence beneficial microbial behavior in the phyllosphere could be used in agriculture to enhance productivity and limit environmental impact while maintaining food safety.

Consideration of the plant's "second genome" could in the future be incorporated into

efforts to improve crop yield and disease resistance (Bakker et al., 2012). Individual microbial taxa associated with plants frequently exhibit several unique plant growth and health promoting characteristics; a more holistic approach to the use of microorganisms in agriculture could be adopted with the use of "plant probiotics," capitalizing upon these stacked characteristics (Berlec, 2012). In the same way that "rhizoengineering" has been suggested, "phylloengineering" could be utilized at the interface between the environment and the aboveground plant surface. Using this approach, a plant's microbiome would be considered as significantly as the plant's own genotype and phenotype.

The potential of phyllosphere microorganisms to reduce the negative environmental impacts of agricultural production is an exciting avenue for future research. Plant breeding efforts could focus on those plants that actively recruit microbes efficient in phytoremediation (Ali et al., 2012). The use of plant growth promoting microorganisms could lead to reduced use of synthetic fertilizers, which in turn would lead to decreased runoff and eutrophication of waterways. Harnessing the ability of microorganisms to stimulate plant defense and antagonize pathogens could lead to reduced use of pesticides, and an associated decrease in human health risk associated with applying and consuming these pesticides. Perhaps microbial communities could even be used to enhance the healing properties or nutritional content of plants; research has shown that phytotherapeutic compounds attributed to medicinal plants are often in fact produced by their associated microbial communities (Koeberl et al., 2013).

Microbial characterization of plants commonly implicated in food safety incidents may assist with the prevention of foodborne outbreaks, as well as increase the speed of trace back in the event of an outbreak. Characterization of microbial communities associated with plants naturally contaminated with enteric pathogens will provide insight into the microbial ecology that allows for the introduction and survival of these pathogens. Indicator species may be identified as precursors or sentinels to *Salmonella* or other pathogen infections. If these indicator species are present in larger amounts than the contaminant, or if they are easier to detect in field labs, they could be valuable resources for prevention and detection of foodborne disease. Tomatoes have frequently been associated with *Salmonella* outbreaks, and at least twice in the past 11 years the outbreak strain has been traced back to on-site irrigation ponds (Greene et al., 2008). California is another major tomato-producing state, and yet out of the 17 outbreaks of *Salmonella* in tomatoes in the US over the past 13 years, only 1 originated in California (Ottesen et al., 2013). Analysis of the microbial communities across these geographical areas may provide clues as to the biological factors that cause the disparity between east coast and west coast food safety risks. Another potential benefit of characterization of microbes associated with at-risk food crops is the discovery of biocontrol organisms. If some organisms seem to be highly enriched in healthy, disease-free plants and deficient in infected plants, these could be investigated for commercial potential as microbial inoculants to encourage plant growth and prevent pathogen infection. Biocontrols for plant pathogens do not appear to have lasting effects on phyllosphere and rhizosphere microbial communities and could represent an environmentally sound approach to foodborne pathogen control in the field (Perazzolli et al., 2014; Sylla et al., 2013).

While the rhizosphere has been studied more extensively than the phyllosphere, the importance of phyllosphere communities and their relationship to crops should not be overlooked. The phyllosphere exists at the interface between the aboveground environment and, in most cases, the marketable portion of crops. There are many avenues for pathogen infection in the phyllosphere, including water, air, soil particles, rain splash, animal vectors, and human workers in the field (Barak and Schroeder, 2012). If the goal of agricultural research is to increase yield and decrease human and plant pathogen infection, the phyllosphere is an essential component to consider for future technological advances. Investigations into how farming practices influence phyllosphere communities at different spatial and temporal scales will allow for better predictive capabilities and risk assessment.

Chapter 3: *Solanum lycopersicum* (tomato) hosts robust phyllosphere and rhizosphere bacterial communities when grown in soil amended with various organic and synthetic fertilizers

1. Introduction

Plants support diverse microbial communities above and belowground that are uniquely suited to the plant habitat and intimately connected to plant health. Microbial communities living on plant surfaces are species- and sometimes genotype-specific (Bulgarelli et al., 2012; Micallef et al., 2009a; Peiffer et al., 2013), and vary across spatial and temporal scales. In the rhizosphere, the region of soil closest to the root structure, root exudates drive composition and structure of bacterial communities, distinguishing them from those of the surrounding bulk soil (Bais et al., 2006; Micallef et al., 2009b). In the phyllosphere, dominated by leaves but also including stem, blossom, and fruit surfaces, harsh and fluctuating environmental conditions present challenges to bacterial epiphytes (Vorholt, 2012). Phyllosphere microbiota may initially be gleaned from air (Fahlgren et al., 2010; Maignien et al., 2014), nearby plants (Vorholt, 2012), or even from seed (Lopez-Velasco et al., 2013) early in life. As the plant develops, the influence of these factors may diminish, with other factors such as plant host and insect visitation becoming more influential (Aleklett et al., 2014; Ushio et al., 2015; Vorholt, 2012). In addition to differing across plant species (Knief et al., 2010; Leff and Fierer, 2013), microbial assemblages vary widely by micro-niche. Within a single tomato plant, leaf, blossom, fruit, stem, and root surfaces hosted unique bacterial and fungal communities, and leaf community diversity decreased with increasing distance from the soil (Ottesen et al., 2013), suggesting that soil may be a source for microbial communities in the

phyllosphere. At an even smaller scale, bacterial communities on specific plant organs may shift in response to nutrient gradients and water availability, such as in close proximity to stomata and leaf trichomes (Leveau and Lindow, 2001; Remus-Emsermann et al., 2012), or on particular floral structures within blossoms (Aleklett et al., 2014). Phyllosphere diversity is consistently lower than in the nutrient-rich rhizosphere, which is not as subject to extreme stresses such as UV exposure and desiccation (Bodenhausen et al., 2013; Ottesen et al., 2013). Both phyllosphere and rhizosphere bacterial community structures shift over time, showing clear successional dynamics throughout growing seasons and plant growth stages (Micallef et al., 2009a; Redford and Fierer, 2009; Shade et al., 2013; van Overbeek and van Elsas, 2008).

While plant host is known to be a strong driving factor of bacterial community composition in the rhizosphere and phyllosphere, the relative contributions of agricultural management practices are less clear. One such management practice is the incorporation of biological soil amendments of animal origin, which are economical, environmentally friendly, and effective sources of soil nutrients for agricultural production. These amendments, including fresh and composted manure, are often incorporated before planting and sometimes used as side dressing throughout the season. In addition to accruing organic matter and improving soil health, biological soil amendments could serve as a source of bacteria for the plant microbiome. Amendments could introduce beneficial microorganisms that directly or indirectly reduce plant pathogen and insect damage (Hadar and Papadopoulou, 2012; Liu et al., 2007; Mehta et al., 2014), or human pathogens that could undermine food safety (Islam et al., 2005; Mootian et al., 2009; Oni

et al., 2015). Investigations into the influence of soil amendment application on plant microbiota have focused primarily on the rhizosphere. Compost or manure has been reported to affect rhizosphere microbial community structure and diversity in some cases (Das and Dhar, 2012; Esperschuetz et al., 2007; Jangid et al., 2008; Lavecchia et al., 2015; Peiffer et al., 2013), but in many studies these effects are inconsistent or seasonally dependent (Gao et al., 2015; Tatti, 2012; Tian and Gao, 2014). On the other hand, the influence of fertilizer application on phytobiomes remains to be studied in depth. Diverse soil microbiomes and soil management strategies cause specific shifts in leaf metabolome composition, and in *Brassica* plants these shifts in turn influenced herbivorous insect damage and diversity of insect pests (Badri et al., 2013; Staley et al., 2010). We hypothesize therefore that amendments applied to the soil may seed the phyllosphere with new microbes, especially in lower parts of the plant close to or touching the ground, and also potentially lead to changes in plant physiology, all of which could influence the diversity of microbes on plant surfaces.

To investigate whether the use of organic fertilization on fields prior to planting can induce above and belowground changes in the harvest-time tomato microbiome, we evaluated synthetic fertilization and three organic fertilizers during tomato cultivation: fresh poultry litter, commercially available sterilized poultry litter pellets, and vermicompost. The impact of these organic fertilizers on tomato rhizobacterial communities, and blossom and fruit-associated bacterial communities was described using Illumina-based 16S rRNA gene sequencing.

2. Materials and Methods

2.1 Field design

Field studies were conducted at the University of Maryland's Wye Research and Education Centre (WyeREC) in Queenstown, MD, USA. In both 2013 and 2014, soil amendments appropriate for both organic and conventional growing operations were applied in spring, 2 weeks prior to planting. In 2013, 4 rows (randomly chosen from a total of 8 rows) were amended with fresh poultry litter mixed from 2 anonymous Eastern Shore sources (Appendix 2, Figure 1). In 2014, research plots were located in the same field, but 5 new rows were prepared between 2013 row locations, so there was no additive effect of amendment application over 2 seasons (Appendix 2, Figure 2). Within each row in 2014, 3 soil amendments and a synthetic fertilizer treatment were assigned randomly and applied to 1 of 4 plots within each row. The following soil amendments were chosen for their ready availability to local growers and for their potentially diverse bacterial profiles: fresh poultry litter (mixed from 2 anonymous local sources), sterile poultry litter pellets (microSTART60, Perdue AgriRecycle, LLC, Seaford, DE), and vermicompost (locally produced from domestic vegetable waste through windrow composting and subsequent digestion by red wriggler worms). One small bag of each soil amendment was transported back to the lab on ice and frozen at -80°C for bacterial community analysis. In both 2013 and 2014, mineral fertilization was applied as an inorganic fertilizer control. Nutrient levels were equalized as much as possible across treatments, using supplemental mineral fertilizer as needed to reach a target nutrition profile of 140N:56P:84K kg/ha). To reduce the probability of drift, rows were spaced 4.6 m apart, with a 1.5 m buffer zone between plots within rows. Field plots were mulched with black plastic and drip irrigated. Treatments had 4 (2013) or 5 (2014) independent

replicate plots, each planted with 8-10 tomato plants. The tomato cultivar used was 'BHN602', a commonly used commercial, indeterminate variety with resistance to Verticillium wilt, Fusarium wilt, and tomato spotted wilt virus. Plants were started from seed in the greenhouse and transplanted into the field after approximately 6 weeks of growth and 2 weeks after soil amendment.

2.2 Sampling for microbial community analysis

Field samples were collected in August 2013 and September 2014, when plants had blossoms and fruit at varying levels of maturity. Three red-ripe fruit, 6-10 blossoms, and the full root ball with adhering soil were randomly sampled from one plant within each plot. Fruit and blossom samples were aseptically collected into Ziploc bags using gloved hands and ethanol-sterilized pruners. After fruit and blossom sampling, each tomato plant was cut at the lower stem and removed from the plot. Soil around the roots was loosened using an ethanol-sterilized trowel, and the roots were manually pulled from the ground with gloved hands. Loosely adhering bulk soil was firmly shaken from the root ball into a Ziploc bag (to be discarded), and the remaining root with closely adhering rhizosphere soil was moved into the final sample bag. Plant samples were transported on ice to the lab, where they were stored at 4°C and processed within 24 h. In 2013 only, bulk soil was collected from each plot for microbial community analysis. For each plot, a composite of 10 soil cores, collected 10 cm from the base of each tomato plant at a depth of 15-20 cm, was collected at planting date in early June using ethanol-sterilized soil corers. Bulk soil samples were transported on ice to the lab, where they were hand-homogenized and frozen at -80°C.

2.3 Plant surface washing, DNA isolation and amplicon sequencing

Aseptically collected samples were washed with sterile deionized water and sonicated for 6 min to dislodge microbial cells from the plant surface. Rhizosphere washes were pelletized at 1773 *g* for 30 min at 4°C using a Sorvall centrifuge with an SA-600 rotor. Blossom and fruit washes were filtered through sterile 0.22 μm nitro-cellulose filters (Nalgene Nunc International Corporation, Rochester, NY). Pellets and filters were frozen at -80°C until further processing. Total community DNA was extracted from frozen rhizosphere pellets and bulk soil samples using the Powerlyzer Powersoil kit (MoBio Laboratories, Carlsbad, CA) and from plant surface-wash filters using the Powerwater kit (MoBio Laboratories). Bacteria represent the most abundant inhabitants of the phyllosphere, therefore community profiling targeted the 16S region of the prokaryotic 30S small ribosomal subunit, which contains both highly conserved and highly variable regions. In 2013, the V4 region of the 16S rRNA gene was amplified using 515F-806R primers as recommended by the Earth Microbiome Project (Caporaso et al., 2012), and libraries were sequenced on an Illumina MiSeq (v2) using 251 bp paired-end sequencing. In 2014, the V1-V3 region of the 16S rRNA gene was chosen with the aim of obtaining a higher resolution within the Enterobacteriaceae, a group with high significance for food safety and plant pathology. This region was amplified using 8F-533R primers (Ottesen et al., 2013), and sequencing was carried out using 300 bp paired-end sequencing on the Illumina MiSeq (v3), following Illumina's protocol for 16S Metagenomic Sequencing Library Preparation (Illumina part # 15044223 rev. B). PhiX (50% in 2013; 25% in 2014) was spiked into each run to provide diversity necessary for cluster generation.

2.4 Sequencing data analysis

Quality filtering and sequence analysis were carried out using QIIME v. 1.8 (Caporaso et al., 2010b) and Mothur v. 1.34 (Schloss et al., 2009). Alignment was performed using PyNAST (Caporaso et al., 2010a) and the Greengenes Core reference alignment (DeSantis et al., 2006; McDonald et al., 2012), and taxonomy assignment utilized the RDP Classifier 2.2 (Wang et al., 2007). Reads that failed to match the reference database were clustered *de novo* using UCLUST (Edgar, 2010). Prior to alignment, sequences went through several quality filtering steps to remove chimeras (Edgar et al., 2011), non-target sequences (chloroplast and mitochondria), and sequences less than 100 bp in length. A final operational taxonomic unit (OTU) table was created excluding unassigned sequences and singletons.

To ensure comparability between samples, within each comparison all samples were rarefied to a common sequencing depth as recommended by Weiss et al. (Weiss et al., 2015). After analyzing the data at several rarefaction depths, numbers were chosen that allowed the inclusion of as many replicates as possible without loss of statistical signal. Plant organ types were analyzed separately to assess the influence of soil amendment treatment and bulk soil properties on microbial diversity for each of these diverse niches. Comparisons across all sample types from 2014 were performed at a depth of 2,450 sequences per sample. When analyzing plant parts separately, the rarefaction level was adjusted: 1,670 for fruit, 1,980 for blossoms, and 5,950 for the rhizosphere. Rhizosphere communities tended to have higher alpha diversity compared to phyllosphere communities, and as such higher thresholds were chosen for rhizosphere samples when

possible. In 2013, rarefaction was employed as follows: 5,570 for soil, 4,545 for rhizosphere, 6,330 for blossom, 3,875 for fruit samples. Comparisons including the full set of samples were carried out with 3,606 sequences per sample..

UniFrac was utilized in QIIME to calculate beta diversity metrics weighted by phylogenetic distance (Chang et al., 2011; Lozupone and Knight, 2005). After filtering to include only OTUs present in at least 75% of samples, as recommended by QIIME documentation, significant differences in relative abundance among OTUs were assessed through a Kruskal-Wallis test utilizing an FDR correction (group_significance.py in QIIME). ANOSIM, an analysis of similarity test, was implemented using R's Vegan package (Oksanen et al., 2013) to assess significance of treatment influence on microbial community structure (999 permutations per test). Pairwise comparisons within treatments were carried out using a 2-sided 2-sample *t*-test of distance through QIIME's make_distance_boxplots.py script. Within the blocking factor of row, the influence of soil amendment treatment was assessed in PC-ORD v.6 (McCune and Mefford, 1999) using 4,999 permutations of a nested PERMANOVA with a Bray-Curtis distance measure at the genus level. Pearson's correlation coefficients were generated to assess soil texture and water activity gradient influence on rhizosphere genus-level beta diversity using PC-ORD. Principal coordinate analysis (PCoA) plots were created to visualize beta diversity across treatments utilizing a weighted UniFrac distance matrix. A tree displaying the differences between plant organs and soil amendments was created using FastTree (Price et al., 2009) and visualized using FigTree v1.4.2 (http://tree.bio.ed.ac.uk/software/figtree/).

Sequencing data were deposited in the NCBI Sequence Read Archive (SRA) database under accession number SRP074759.

2.5 Soil properties

In 2014, bulk soil was collected from the top 10 cm of each plot using ethanol-sterilized scoops. Soil samples were sent to Waters Agricultural Lab, Inc. (Camilla, GA) for analysis. The following soil properties were measured for soil from each plot: available P, exchangeable K, Mg, Ca, and H, soil pH, cation exchange capacity (CEC), percent base saturation of cation elements, organic matter, and soil texture (% sand, silt, and clay). Water activity (A_w) was assessed using a Pa_wkit water activity meter (Aqualab, Pullman, WA). Significant differences between treatments were assessed using ANOVA and pairwise comparisons utilized Tukey's HSD test (JMP Pro v.11).

3. Results

3.1 Sequencing run metrics

Sequencing of samples collected over two years was performed separately. Sequencing of 2014 samples resulted in 9.3 million total sequences for each of the forward and reverse reads (for the 60 samples taken through the full analysis). Reads 1 and 2 were merged at an average efficiency of 58%. For all unmerged read pairs, read 1 was included for further analysis. After removing low quality or non-target sequences (less than 100 bp long, sequences identified as phiX, chimeric, chloroplast, or mitochondrial), 3.4 million reads remained for OTU picking. For 2013 samples, sequencing on the MiSeq v2 platform resulted in 1.4 million total sequences for the 56 samples included in this study.

Merging efficiency was 84%, and after quality filtering 1.3 million sequences remained for further analysis.

3.2 Plant organ as a driver of bacterial communities

Principal Coordinates Analysis (PCoA) performed through QIIME and analysis by R-Vegan function ANOSIM showed that rhizosphere, blossoms, and fruit supported distinct bacterial communities, with the greatest distance observed between rhizosphere soil and the two phyllosphere groups (Figure 1). Plant organ drove variation in bacterial community structure more than any other factor in 2013 (R=0.87, p=0.001, n=56) and 2014 (R=0.93, p=0.001, n=60). When 2013 and 2014 data were analyzed together, sample type (soil, rhizosphere, blossom, fruit) consistently explained the majority of variation among samples (R=0.69, p=0.001, n=116). Year also had a significant, albeit weaker, effect on bacterial community composition (R=0.37, p=0.001, n=116).

At the phylum level, the largest difference between above- and below-ground bacterial communities was observed in the Proteobacteria, which were much more dominant on fruit and blossoms compared to bulk soil and rhizosphere in both 2013 and 2014. In 2013, blossom and fruit surfaces were dominated by Pseudomonadaceae (50% on blossom and 40% on fruit) and Enterobacteriaceae (39% and 26%, respectively). Dominant taxa in rhizobacterial communities in 2013 belonged to the Bacillaceae (13.6%) and Pseudomonadaceae (12.5%), both of which were highly enriched compared to the surrounding bulk soil. In 2014, Pseudomonadaceae were elevated in blossoms compared to roots, however they were much less prevalent than in 2013, at 9% relative abundance. Instead, Xanthamonadaceae dominated on blossoms (32.6%), while fruit

supported a high relative abundance of Rhizobiaceae (14.3%), mostly explained by the genus *Agrobacterium*, at 13.6%. Both the Pseudomonadaceae and Xanthamonadaceae families contain pathogens that can infect tomato; it is possible that these pathogens occurred in the field, however resolution is not high enough to differentiate between pathogenic and non-pathogenic members of this taxa.

Members of the Paenibacillaceae, a group known to include several bacteria with biocontrol activity against plant and human pathogens, were detected in all sample types over both years, with highest prevalence in the rhizosphere (~1% relative abundance both years). The rhizosphere samples harbored the highest phylogenetic diversity, with an average of 1,764 unique OTUs (97% identity) identified at a rarefaction level of 2,450 sequences per sample in 2014. Alpha (within sample) diversity in blossoms and fruit was significantly lower, with 357 and 693 OTUs identified at the same rarefaction level, respectively (p=0.003) in 2014.

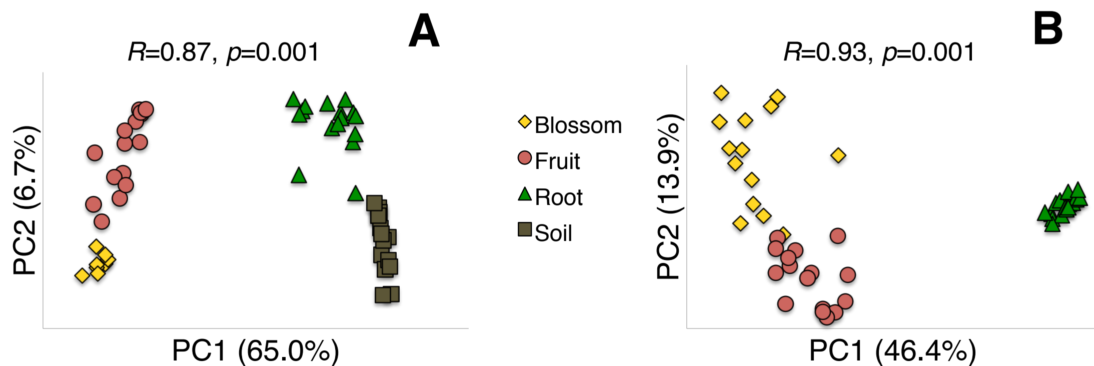


Figure 1. Principal Coordinates Analysis (PCoA) illustrating differences in bacterial community structure on the surfaces of tomato blossoms, fruit, rhizosphere, and soil in 2013 (A) and 2014 (B). A distance matrix weighted by abundance and utilizing UniFrac

distances was used to calculate principle coordinates. Percent variation explained by each principle coordinate is marked on each axis. For each year, the strength of the sample type grouping is denoted by p values for the ANOSIM R statistic, representing the strongest correlation as it approaches 1.

3.3 Influence of soil amendment on tomato-associated bacterial communities

The data from 2013 indicated a potentially weak influence of poultry litter amendment on tomato blossom (R=0.34, p=0.076, n=8) and rhizosphere (R=0.16, p=0.051, n=16), but not fruit (R=0.04, p=0.319, n=16), bacterial communities (Figure 2). Bacterial communities profiled from bulk soil did not respond to soil amendment (R=0.02, p=0.293, n=16), however row location in the field appeared to influence bulk soil bacterial community structure (R=0.58, p=0.002, n=16).

In 2014, to better control for the confounding influence of soil parameters and strengthen the study design, the field was blocked by row (with all treatments incorporated into plots within each row). We were also able to introduce greater replication and two more soil amendment treatments. Despite these changes and the use of a longer 16S rRNA gene fragment for sequencing, no effect of soil amendments on the tomato microbiome was observed in 2014. Soil amendment was not a significant factor for bacterial community structure in the rhizosphere (Figure 2), and no significant differences were observed at any taxonomic level. Likewise, in 2014, blossom and fruit surfaces hosted convergent bacterial communities across soil amendment treatments (Figure 2). When analyzed within field row in a nested perMANOVA utilizing a Bray-Curtis distance measure, soil amendment had no influence on 2014 bacterial community structure on any tomato plant

surface studied (*p*>0.05). To verify that the 2013 detection of a weak soil amendment treatment effect on blossom-associated bacterial community structure was not attributable to a higher sampling depth in 2013, 2013 blossom data was reanalyzed at the 2014 rarefaction level of 1,980 sequences per sample. The weak effect of poultry litter application on blossom microbiome remained (R=0.34, p=0.095, n=8).

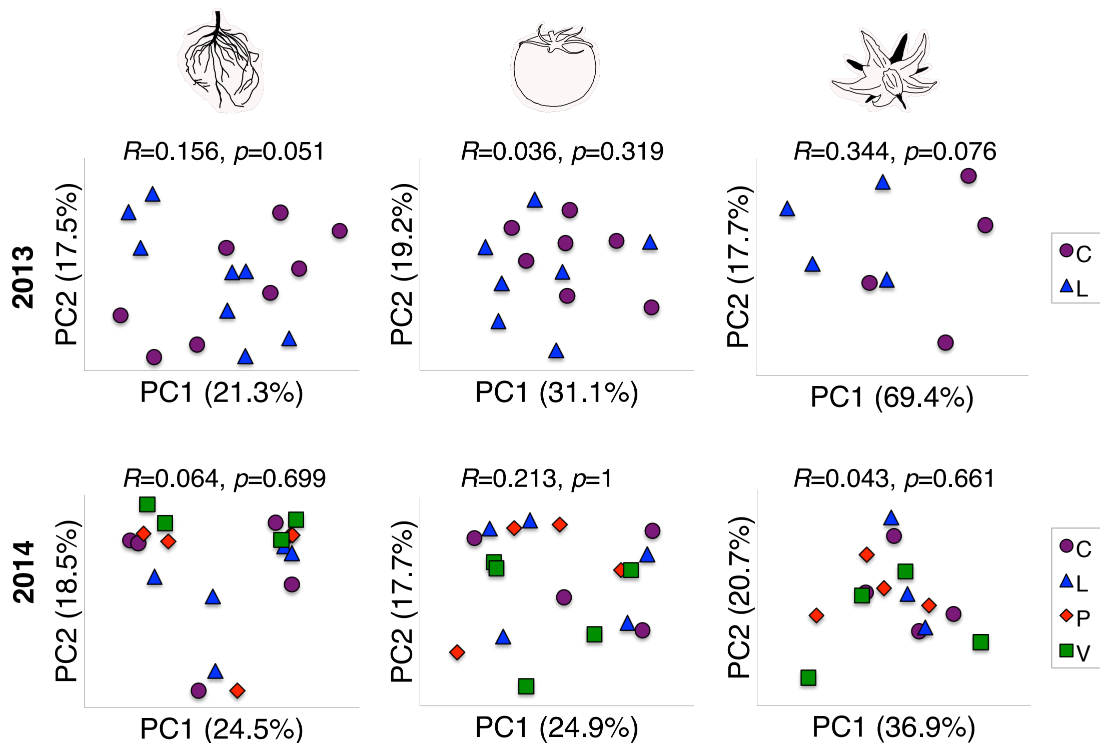


Figure 2. Influence of soil amendment application on tomato plant surface-associated bacterial communities at time of tomato harvest in 2013 (top) and 2014 (bottom). Principle Coordinates Analysis utilizing a distance matrix weighted by OTU abundance and UniFrac distance between related taxa was performed to compare the beta diversity between tomato rhizosphere, fruit, and blossom bacterial communities from plots amended with poultry litter (L), poultry pellets (P), vermicompost (V), or mineral

nutrition (C). To compare similarity between groups, *R* and *p* values were generated using ANOSIM. As *R* approaches 1, samples collected from plots treated with the same soil amendments are more similar to each other than to those collected from differently amended plots.

3.4 Microbiome analysis of organic soil amendments

The soil amendments themselves supported phylogenetically diverse bacterial assemblages ($p<0.001$, UniFrac Monte Carlo significance test). Vermicompost harbored a highly diverse bacterial community most similar to that of the rhizosphere, while the poultry litter and poultry pellets were characterized by a less diverse community dominated by several shared families (Figure 3). Dominant taxa in vermicompost were Hyphomicrobiaceae, Acidimicrobiales and Bacillaceae. On the other hand, the most predominant groups in poultry manure and poultry litter pellets were the Staphylococcaceae, Dermabacteraceae, Lactobacillaceae and Aerococcaceae (Figure 3). While bacterial assemblages in poultry pellets were most similar to those in fresh poultry litter, a large proportion of the DNA isolated from the former samples could likely have persisted from non-viable organisms killed during the sterilization process.

Despite differences in bacterial communities among these diverse soil amendments, a significant differential shift in bacterial community structure or diversity in the mature plant rhizosphere or phyllosphere at harvest was not observed. Additionally, application of diverse soil amendments did not lead to changes in most physico-chemical soil characteristics, the only measurable difference being an elevated Cation Exchange Capacity (CEC) in vermicompost-amended plots (Table 1).

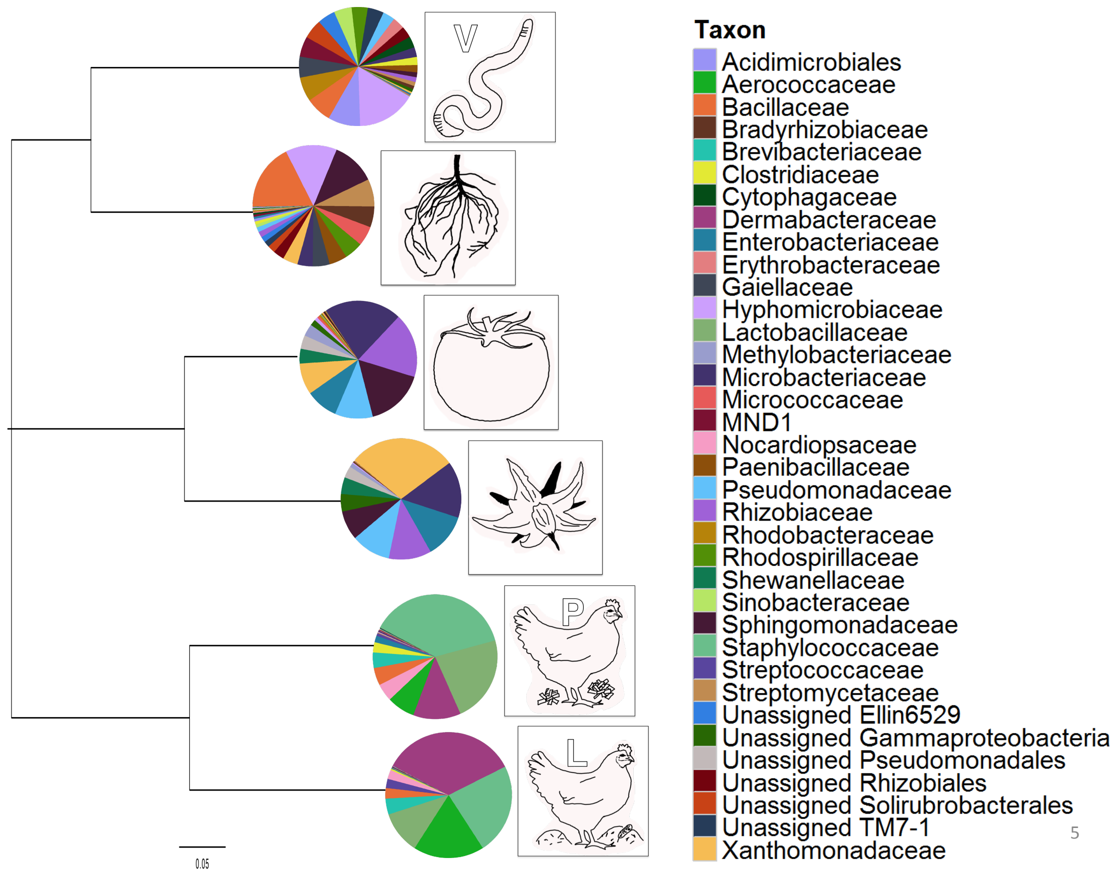


Figure 3. Phylogenetic relationship of tomato plant organ surfaces and starting soil amendments. The top 20 most abundant families within each sample type are shown, scaled up to 100%, at the tips of the tree. The tree was created in FastTree and R utilizing UniFrac distances.

Table 1. Differences in soil characteristics among 2014 tomato plots considering application of different soil amendments and field location by row and column, as determined by ANOVA (*p* values are shown).

Soil Factor	Soil Amendment[a]	Row[b]	Column[b]	Trends
Sand (%)	0.399	0.002*	0.752	Decreasing from row 1 to 5
Clay (%)	0.678	0.108	0.642	
Silt (%)	0.572	0.0003*	0.574	Increasing from row 1 to 5
pH	0.414	0.408	0.35	
Organic Matter (%)	0.178	0.435	0.456	
CEC (meq/100g)	0.031*	0.713	0.491	Highest in vermicompost plots
Water Activity (A_w)	0.794	0.084	0.297	Highest in rows 4 and 5
P (kg/ha)	0.343	0.488	0.315	
K (kg/ha)	0.991	0.401	0.021*	Increasing from column 1 to 4
Ca (kg/ha)	0.207	0.193	0.976	
ENR (kg/ha)	0.224	0.382	0.469	
Mg (kg/ha)	0.061	0.018*	0.983	Higher in row 5 than 2 and 3; Higher in amended plots compared to controls

[a]Blocked by row
[b]Blocked by soil amendment
*$p<0.05$

3.5 Impact of edaphic factors on tomato-associated bacterial communities

While soil amendment application did not exert a drastic influence on tomato-associated bacterial communities, soil physico-chemical characteristics may have played a role. Rhizosphere samples taken from field rows 4 and 5 supported phylogenetically similar bacterial communities, as did those from rows 2 and 3, and both pairs differed from row 1, regardless of soil amendment treatment applied (Figure 4B).

Analysis of bulk soil collected from all plots showed a clear gradient in soil texture ($p<0.001$) and Mg concentration ($p=0.016$) through the field, coupled with a weakly significant gradient in water activity ($p=0.080$) (Table 1, Figure 4A). Shifts in rhizosphere beta diversity were correlated with continuous silt ($R^2=0.52$, $p=0.005$) and inversely correlated with sand ($R^2=0.48$, $p=0.009$) gradients in the field. A weak association with the water activity gradient was also observed but was not statistically supported ($R^2=0.12$, $p=0.14$). These soil characteristics could be important factors affecting bacterial community structure in the rhizosphere; both water availability (Fierer et al., 2003; Reichel et al., 2014) and soil texture (Schreiter et al., 2014) have been previously identified as drivers of rhizosphere community structure, and both of these factors likely modulate the availability of nutrients such as Mg.

While row and its associated soil characteristics tended to influence beta diversity both in the rhizosphere and on ripe fruit surfaces (Figure 4B), statistically significant differences were observed at the family level only in the rhizosphere (Figure 5). Rhizosphere soil collected from rows 4 and 5 hosted lower percentages of Bacillaceae and Mycobacteriaceae and higher percentages of Oxalobacteriaceae and Pseudomonadaceae compared to the other rows. Shifts in fruit-associated beta diversity differed by row as well, with samples from rows 1 and 5 clustering together and rows 3 and 4 forming another cluster, with both clusters diverging from row 2. While row did not significantly drive blossom-associated bacterial beta diversity, rows 3 and 4 tended to cluster more closely together, as did rows 1 and 2.

A closer look at the relative abundance of taxa across rows in each sample type revealed that common trends in beta diversity across plant organs could not be attributed to shifts in the abundance of the same taxa (Figure 5). Although levels of Xanthomonadaceae were consistent across all rhizosphere samples, relative abundance appeared to be slightly higher on blossoms, increasing by at least 20% in rows 4 and 5 compared to the rest of the field (although this variation was not significant). On fruit, Xanthomonadaceae was highest in rows 3 and 4 compared to the rest of the field, with row 5 being lowest. While row was used as a blocking factor throughout sample processing, no other factor (placement on PCR plate, DNA extraction date/lot of kit, indexing primers used) had a significant influence on beta diversity. Location in the field based on the perpendicular gradient (column) also had no significant effect on any sample type, and only one soil characteristic, K, differed significantly between columns in the field (Table 1).

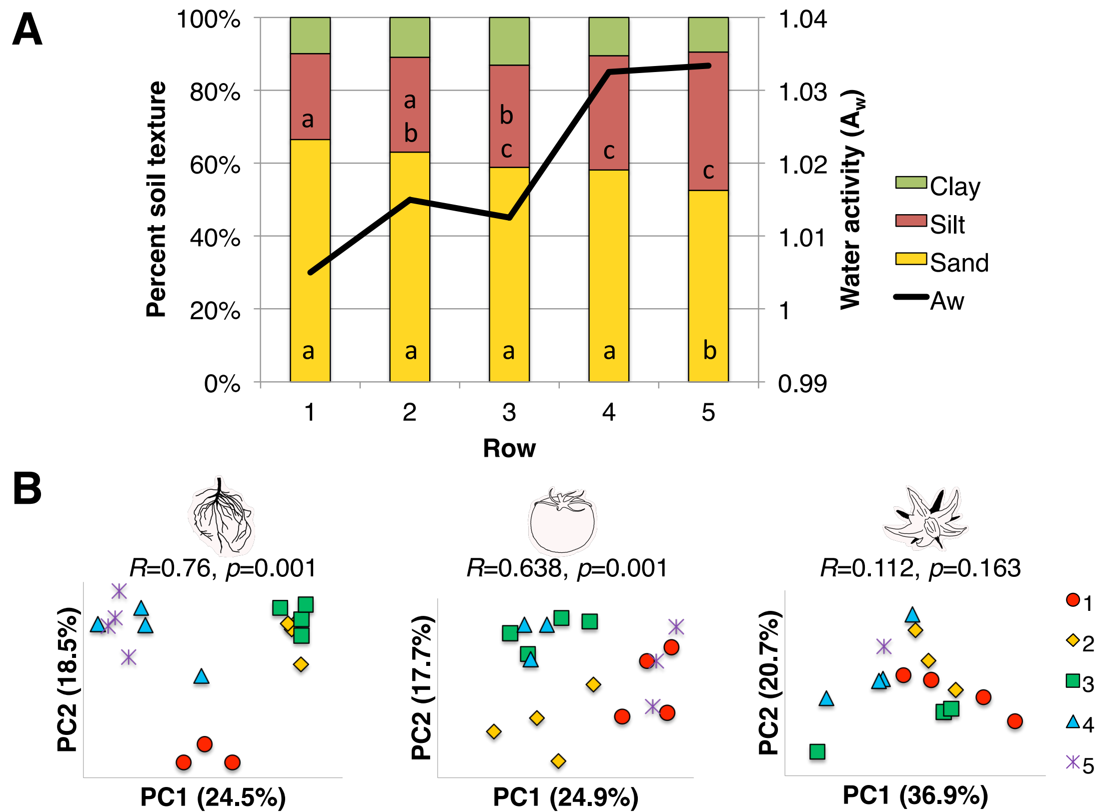
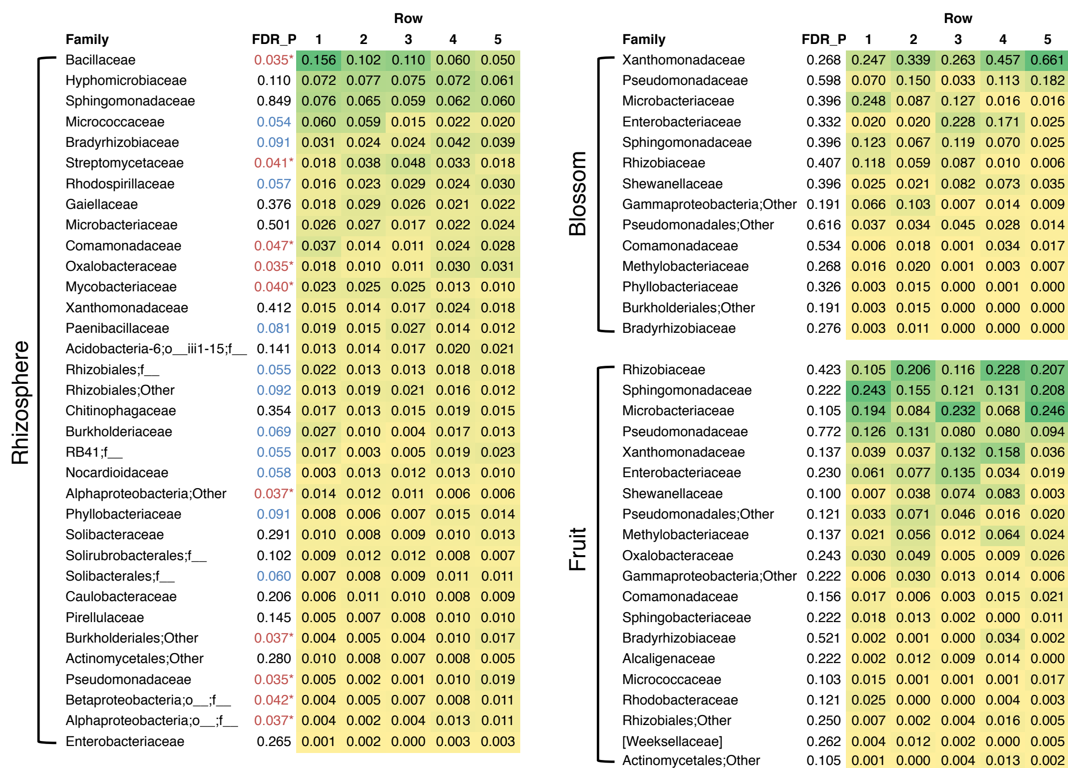


Figure 4. Soil texture (bars) and water activity (line graph) by tomato row in the field (A), and within plant organ beta diversity by row, represented by PCoA plots (B). Letters in bars representing sand and silt fractions in panel (A) denote significant differences in those soil components (p<0.05). Legend in panel (B) shows symbols for rows 1-5. R and p values were calculated through ANOSIM using a Bray-Curtis distance metric. As R approaches 1, samples within a row are more similar to each other than to samples from other rows.



Rhizosphere Family	FDR_P	Row 1	Row 2	Row 3	Row 4	Row 5
Bacillaceae	0.035*	0.156	0.102	0.110	0.060	0.050
Hyphomicrobiaceae	0.110	0.072	0.077	0.075	0.072	0.061
Sphingomonadaceae	0.849	0.076	0.065	0.059	0.062	0.060
Micrococcaceae	0.054	0.060	0.059	0.015	0.022	0.020
Bradyrhizobiaceae	0.091	0.031	0.024	0.024	0.042	0.039
Streptomycetaceae	0.041*	0.018	0.038	0.048	0.033	0.018
Rhodospirillaceae	0.057	0.016	0.023	0.029	0.024	0.030
Gaiellaceae	0.376	0.018	0.029	0.026	0.021	0.022
Microbacteriaceae	0.501	0.026	0.027	0.017	0.022	0.024
Comamonadaceae	0.047*	0.037	0.014	0.011	0.024	0.028
Oxalobacteraceae	0.035*	0.018	0.010	0.011	0.030	0.031
Mycobacteriaceae	0.040*	0.023	0.025	0.025	0.013	0.010
Xanthomonadaceae	0.412	0.015	0.014	0.017	0.024	0.018
Paenibacillaceae	0.081	0.019	0.015	0.027	0.014	0.012
Acidobacteria-6;o__iii1-15;f__	0.141	0.013	0.014	0.017	0.020	0.021
Rhizobiales;f__	0.055	0.022	0.013	0.013	0.018	0.018
Rhizobiales;Other	0.092	0.013	0.019	0.021	0.016	0.012
Chitinophagaceae	0.354	0.017	0.013	0.015	0.019	0.015
Burkholderiaceae	0.069	0.027	0.010	0.004	0.017	0.013
RB41;f__	0.055	0.017	0.003	0.005	0.019	0.023
Nocardioidaceae	0.058	0.003	0.013	0.012	0.013	0.010
Alphaproteobacteria;Other	0.037*	0.014	0.012	0.011	0.006	0.006
Phyllobacteriaceae	0.091	0.008	0.006	0.007	0.015	0.014
Solibacteraceae	0.291	0.010	0.008	0.009	0.010	0.013
Solirubrobacterales;f__	0.102	0.009	0.012	0.012	0.008	0.007
Solibacterales;f__	0.060	0.007	0.008	0.009	0.011	0.011
Caulobacteraceae	0.206	0.006	0.011	0.010	0.008	0.009
Pirellulaceae	0.145	0.005	0.007	0.008	0.010	0.010
Burkholderiales;Other	0.037*	0.004	0.005	0.004	0.010	0.017
Actinomycetales;Other	0.280	0.010	0.008	0.007	0.008	0.005
Pseudomonadaceae	0.035*	0.005	0.002	0.001	0.010	0.019
Betaproteobacteria;o__;f__	0.042*	0.004	0.005	0.007	0.008	0.011
Alphaproteobacteria;o__;f__	0.037*	0.004	0.002	0.004	0.013	0.011
Enterobacteriaceae	0.265	0.001	0.002	0.000	0.003	0.003

Blossom Family	FDR_P	Row 1	Row 2	Row 3	Row 4	Row 5
Xanthomonadaceae	0.268	0.247	0.339	0.263	0.457	0.661
Pseudomonadaceae	0.598	0.070	0.150	0.033	0.113	0.182
Microbacteriaceae	0.396	0.248	0.087	0.127	0.016	0.016
Enterobacteriaceae	0.332	0.020	0.020	0.228	0.171	0.025
Sphingomonadaceae	0.396	0.123	0.067	0.119	0.070	0.025
Rhizobiaceae	0.407	0.118	0.059	0.087	0.010	0.006
Shewanellaceae	0.396	0.025	0.021	0.082	0.073	0.035
Gammaproteobacteria;Other	0.191	0.066	0.103	0.007	0.014	0.009
Pseudomonadales;Other	0.616	0.037	0.034	0.045	0.028	0.014
Comamonadaceae	0.534	0.006	0.018	0.001	0.034	0.017
Methylobacteriaceae	0.268	0.016	0.020	0.001	0.003	0.007
Phyllobacteriaceae	0.326	0.003	0.015	0.000	0.001	0.000
Burkholderiales;Other	0.191	0.003	0.015	0.000	0.000	0.000
Bradyrhizobiaceae	0.276	0.003	0.011	0.000	0.000	0.000

Fruit Family	FDR_P	Row 1	Row 2	Row 3	Row 4	Row 5
Rhizobiaceae	0.423	0.105	0.206	0.116	0.228	0.207
Sphingomonadaceae	0.222	0.243	0.155	0.121	0.131	0.208
Microbacteriaceae	0.105	0.194	0.084	0.232	0.068	0.246
Pseudomonadaceae	0.772	0.126	0.131	0.080	0.080	0.094
Xanthomonadaceae	0.137	0.039	0.037	0.132	0.158	0.036
Enterobacteriaceae	0.230	0.061	0.077	0.135	0.034	0.019
Shewanellaceae	0.100	0.007	0.038	0.074	0.083	0.003
Pseudomonadales;Other	0.121	0.033	0.071	0.046	0.016	0.020
Methylobacteriaceae	0.137	0.021	0.056	0.012	0.064	0.024
Oxalobacteraceae	0.243	0.030	0.049	0.005	0.009	0.026
Gammaproteobacteria;Other	0.222	0.006	0.030	0.013	0.014	0.006
Comamonadaceae	0.156	0.017	0.006	0.003	0.015	0.021
Sphingobacteriaceae	0.222	0.018	0.013	0.002	0.000	0.011
Bradyrhizobiaceae	0.521	0.002	0.001	0.000	0.034	0.002
Alcaligenaceae	0.222	0.002	0.012	0.009	0.014	0.000
Micrococcaceae	0.103	0.015	0.001	0.001	0.001	0.017
Rhodobacteraceae	0.121	0.025	0.000	0.000	0.004	0.003
Rhizobiales;Other	0.250	0.007	0.002	0.004	0.016	0.005
[Weeksellaceae]	0.262	0.004	0.012	0.002	0.000	0.005
Actinomycetales;Other	0.105	0.001	0.000	0.004	0.013	0.002

Figure 5. Mean relative abundance of families identified from tomato rhizosphere, fruit, and blossom surfaces from rows 1-5 in 2014. Families with a relative abundance greater than 0.01 for at least one row within each tomato organ are shown (after filtering to OTUs present in >25% of each sample type). FDR-corrected p values from a Kruskal-Wallis test are given for differences within a row. Relative abundances are coded by color, with the highest abundances marked in dark green and the lowest abundances marked in light yellow.

4. Discussion

Using a phylogenetic approach, this study found that the application of three locally available organic soil amendments—fresh poultry litter, sterile poultry litter pellets, and

vermicompost—did not exert a remarkable differential influence from synthetic fertilizer on tomato rhizosphere, blossom or fruit-associated bacterial communities when applied before planting. At harvest time, tomato plants supported bacterial communities that were plant organ-specific but generally independent of soil amendment. This finding indicates that tomato plants are robust hosts to epiphytic bacteria with the ability to maintain a consistent selective pressure on plant-associated microbiota, despite changing agricultural inputs. Although we observed no effect attributable to soil amendment, our study was limited to one time point, and differences in plant-associated microbiomes in response to soil amendment may have existed earlier during plant development. Studies of the cucumber and bean rhizosphere found that microbial community response to compost amendments was strong during early plant establishment but decreased throughout the season as plants matured (Copeland et al., 2015; Gao et al., 2015). This suggests that over time plant-mediated pressures override the influence exerted by the soil amendments, however, additional research is needed to determine the contribution of such agricultural inputs to the crop microbiome early in plant development.

Above-ground, the use of diverse organic soil amendments did not lead to consistent changes in microbial community structure or relative abundance of bacterial taxa. In 2013, the application of poultry manure may have shifted blossom- and rhizosphere-associated bacterial community structure but not relative abundance of specific taxa. This effect was not detected in 2014. Weather patterns and field management practices were similar between 2013 and 2014 sampling periods, but the field setup in the first year of sample collection did not include replication within rows. It is possible that the difference

in effect could be attributed to field gradients, or other factors that we did not measure. In any case, our findings do suggest that interactions with one or more unidentified factors may occur that could augment the influence of the organic fertilizers used in this study.

Although fertilizer type appeared to be a less significant factor than expected, by contrast, location in the field exerted a measurable effect on crop-associated bacterial assemblages. Statistically significant increases in silt and sand content across the field from rows 1 to 5 were associated with gradual changes in soil water activity, which in turn were paralleled with shifts in rhizobacterial community structure. Relative abundance measures of some taxa were significantly different across these soil characteristic gradients. Previous work has reported the effect of edaphic factors on the rhizosphere. Soil organic matter was a factor for cucumber rhizobacterial community structure, whereas compost use exerted no long-term impact (Tian and Gao, 2014). On the other hand, a study of the grape microbiome showed that the use of compost over several years, leading to changes in physico-chemical soil characteristics, did not induce changes in the rhizosphere microbiome (Tatti, 2012). Shifts in phyllosphere bacterial communities from different rows were less dramatic compared to rhizosphere samples, with significant differences in beta diversity but not relative abundances of bacterial taxa observed. Soil conditions, such as C:N ratio and pH have previously been shown to influence phyllosphere microbial community structure (Zarraonaindia et al., 2015). Our results support this trend, although we observed a weaker response in the phyllosphere compared to the rhizosphere. Fruit surface bacterial community groupings somewhat followed the gradient of increasing water activity and changing soil texture from rows 1 to 5, but

additional associations were noted, showing that at least one additional factor (again associated with field location) was a strong driver of the tomato microbiome above-ground. Microbial communities collected from fruit surfaces from rows 1 and 5 were statistically similar, sharing a high prevalence of certain families such as Sphingomonadaceae and Microbacteriaceae. While border rows were employed in the study design to attenuate any edge effect, rows 1 and 5 were still closest to the edge of the field, which was flanked on each side by roads used by farm vehicles. Dust from passing vehicles may have influenced microbial diversity on fruit surfaces on the outer rows, causing them to host similar bacterial communities despite different soil conditions.

In this study, soil amendments were applied to supplement existing bulk soil nutrition as an alternative to synthetic nutrition alone. Manure- or compost-based amendments may be chosen for nutrient management in organic or conventional growing operations due to their widespread availability, affordable cost, and effectiveness in releasing nutrients slowly throughout the season. Many organic growers use animal-derived fertilizer (fresh or composted manure) as a primary source of plant nutrition, and it has been posited that organically grown produce could therefore have a higher risk of contamination with enteric human pathogens. Many consumers on the other hand assume that organically grown produce is "safer" than its conventional counterpart (Berlin et al., 2009; Williams and Hammitt, 2001). In actual fact, studies do not tend to support this – many studies comparing the microbiological safety of conventional versus organic produce tend to show no differences in microbiological safety risk (Bourn and Prescott, 2002; Diez-Gonzalez and Mukherjee, 2009; Magkos et al., 2006; Marine et al., 2015; Pagadala et al.,

2015). Many of these studies have used bacterial indicators of fecal contamination, such as generic *E. coli* and fecal coliforms, to assess risk, however these indicators have been shown to have little to no correlation with the presence of pathogens (Pachepsky et al., 2014; Wu et al., 2011). Better and more comprehensive methods are needed to assess the relative risk of agricultural management practices, including use of manure, on produce safety.

By understanding the ecological influence of biological soil amendment use on plant-associated microbial communities, we will come closer to understanding how certain nutrient management practices influence food safety risk in agriculture. Samples of soil amendments and rhizosphere soil, blossoms and fruit were analyzed for the foodborne pathogens *Salmonella enterica* and *Listeria monocytogenes* (data not shown). No foodborne pathogens were detected from the soil amendments used in this study, so the potential for transmission to the field and survival throughout the season could not be assessed. Instead, we investigated the potential for soil amendment application to directly or indirectly influence the makeup of bacterial assemblages in the tomato rhizosphere, and on blossom and fruit surfaces, finding that location of the plant in the field and plant organ were much more influential.

5. Conclusions

Investigating the impact of soil edaphic characteristics on the tomato microbiome was not the aim of this study, but the effect of row (and its associated soil texture and water activity gradients) was notable, especially in contrast to the apparent lack of influence of

soil amendments. While the plant host itself, and the organ-specific niches it provides, regulated bacterial community structure to a large extent, this study showed that field location and associated soil characteristics had a stronger influence than poultry litter fertilizer or vermicompost. The effect was more marked belowground, but certain shifts were also observed in phyllosphere communities. This study suggests therefore that in the short term, poultry litter-based manure and vermicompost amendments applied to soil before transplanting of seedlings are not important determinants of the tomato microbiome at the time of harvest. On the other hand, location in the field, which may be subject to variable environmental conditions such as changes in soil characteristics or air quality, may be important factors to evaluate. This segues to possible effects of long-term organic fertilization, which tends to build organic matter over time and alters physical characteristics, which would be expected to exert important influences. Long–term studies are needed to test this hypothesis, determine whether such changes are also specific to plant developmental stage, and how these complex factors contribute to crop health and safety.

Chapter 4: Response of tomato and cucumber epiphytic microbiomes to rainfall

1. Introduction

Microbiome analyses of fruits and vegetables are revealing diverse bacterial assemblages associating with various plant organs (Jackson et al., 2013; Leff and Fierer, 2013; Ottesen et al., 2013). Plant microbiomes are dynamic and undergo successional changes with plant development (Micallef et al., 2009a), possibly with new introductions occurring throughout the plant life cycle. Several bacterial reservoirs for the phyllosphere microbiome have been reported, including the air (Vorholt, 2012), insect pollinators (Ushio et al., 2015), seed (Lopez-Velasco et al., 2013), other nearby plants (Vorholt, 2012), and meteorological conditions (Rastogi et al., 2012). The impact of the latter on fresh produce crop microbiomes is of particular interest, due to the highly variable nature of weather-related events, variation due to geography, and anticipated changes in precipitation patterns in the coming years due to climate change (Huntington, 2006). Increased rainfall and humidity often favor the development of plant disease (Lamichhane et al., 2015; Thompson et al., 2013). Similarly, the prevalence of several foodborne pathogens including pathogenic *Escherichia coli*, *Campylobacter jejuni*, *Salmonella enterica*, and *Bacillus cereus* has been correlated with elevated environmental temperature and humidity (Kim et al., 2015; Semenza et al., 2012). In trials assessing the fate of *E coli*, fecal coliforms and enterococci applied to the lettuce phyllosphere, bacterial decline rates were slower under moderate and regular rain patterns, and faster following a heavy rain event (Xu et al., 2016). At the community level, one study suggested that rainfall events may coincide with drastic changes in the leaf surface

microbiomes of canola plants, although changes due to plant development could not be detangled in this study (Copeland et al., 2015). Some soil microbial communities are influenced by drying and wetting frequencies, especially those not normally exposed to large fluctuations in soil moisture (Bapiri et al., 2010; Fierer et al., 2003).

Rainfall may shift the microbial profile of phyllosphere communities through direct seeding of microbes present in rainwater, splash from surrounding soil, increasing water availability, or by washing off loosely adhered microbes, creating opportunities for others to fill their former niche. Airborne biological particles, including bacteria and fungi, may act as ice or cloud nuclei, particles around which rain droplets form. Levels of bioaerosols are elevated during rain events (Huffman et al., 2013), and in fact plants have been suggested as "cloud seeders" (Morris et al., 2004). Airborne microbes, classified as bioaerosols, may be transferred to plant surfaces directly via rainfall or indirectly from standing water after rainfall. *Salmonella enterica* serovar Typhimurium is capable of aerosolizing from puddles and colonizing tomato plants following simulated rain events (Cevallos-Cevallos et al., 2012b). Rain splash dispersal can facilitate the transfer of human enteric bacteria from bulk soil to leaf and fruit surfaces (Monaghan and Hutchison, 2012) even with the use of plastic mulch as a barrier (Cevallos-Cevallos et al., 2012a). Other microbes including plant pathogens are similarly capable of aerosolizing and retaining viability, sometimes incorporating aerosolization as part of their lifecycle (Morris et al., 2004; Morris et al., 2008). To garner a more comprehensive understanding of the impact of rain on the phytobiome of fresh produce crops that are vulnerable not only to plant disease but also colonization by human pathogens, we characterized the epiphytic bacterial communities dwelling on two commercially important fresh produce

crops. A temporal assessment of the epiphytic microbiomes of commercially cultivated tomato fruit (carpoplane) and leaves (phylloplane) surrounding two rain events and cucumber carpoplane surrounding one rain event was conducted.

2. Materials and Methods

2.1 Sample collection

Samples were collected from an established Maryland farm, under agricultural cultivation for 40 years. Tomato (*Solanum lycopersicum* cultivar 'Christa') and cucumber (*Cucumis sativus* cultivar 'Sweet Success') plants were grown on black plastic mulch and drip irrigated throughout the season. Pest control and fertilizer management were conducted according to typical management practices for the farm. Samples were collected in September 2015, on 5 dates surrounding 2 rain events. At each sampling, tomato fruit (n=7, 3 fruits per sample) and leaves (n=7, 2 compound leaves per sample) and cucumber fruit (n=8, 1 fruit per sample, first 3 dates only) were aseptically collected in Ziploc bags and transported on ice in a cooler to the lab, where they were stored at 4°C until processing on the following day.

2.2 DNA isolation and amplicon sequencing

Sterile deionized water was added to sample bags. Submerged samples were hand massaged through the bag for 30 s then sonicated for 3 min to dislodge bacterial cells from the carpoplane and phylloplane. Samples were hand massaged again and sonicated for an additional 3 min before filtration. Carpoplane and phylloplane washes were filtered through sterile 0.22 µm nitro-cellulose filters (Nalgene, Rochester, NY), and filters were frozen at -80°C until further processing. Total community DNA was extracted from

filters using the MoBio Powerwater kit (MoBio Laboratories, Carlsbad, CA). The V1-V3 region of the 16S rRNA gene was chosen for use in bacterial community profiling because preliminary use suggests a high resolution within Enterobacteriaceae, a group with high significance for food safety and plant pathology. This region was amplified using 8F-533R primers (Ottesen et al., 2013), and sequencing was carried out using 300-bp paired-end sequencing on the Illumina MiSeq (v3). Illumina's protocol for 16S Metagenomic Sequencing Library Preparation was followed for all samples (Illumina part # 15044223 rev. B). PhiX (25%) was spiked into each run to provide diversity necessary for cluster generation.

2.3 Sequencing data analysis

Quality filtering and sequence analysis were carried out using QIIME v. 1.8 (Caporaso et al., 2010b) and Mothur v. 1.34 (Schloss et al., 2009). Prior to alignment, sequences went through several quality filtering steps to remove chimeras (Edgar et al., 2011), non-target sequences (chloroplast and mitochondria), and sequences less than 100 bp in length. Sequences were aligned to the Greengenes Core Set (DeSantis et al., 2006; McDonald et al., 2012) using PyNAST (Caporaso et al., 2010a), and taxonomy assignment utilized the RDP Classifier 2.2 (Wang et al., 2007). Reads that failed to match the reference database were clustered *de novo* using UCLUST v. 1.2.22 (Edgar, 2010).

To ensure comparability between samples, sample datasets within each comparison were subsampled to the lowest common sequencing depth (Weiss et al., 2015). Sample types (cucumber fruit, tomato fruit, tomato leaves) were analyzed separately to assess the influence of rainfall events on bacterial diversity for each of these niches. The rarefaction

level was adjusted for each sample type to: 28,685 for cucumber fruit, 15,440 for tomato fruit, and 17,375 for tomato leaves. For comparisons of all sample types, rarefaction was set at 15,440.

Beta (between sample) diversity was assessed using both unweighted distance matrices and matrices weighted by relative taxon abundance. Phylogenetic distance was incorporated into both distance matrices using UniFrac (Chang et al., 2011; Lozupone and Knight, 2005), both produced in QIIME. Adonis (999 permutations), a nonparametric MANOVA from R's Vegan package, was implemented to assess significance of treatment influence on bacterial community structure. A 2-sided 2-sample *t*-test was employed for pairwise comparisons of bacterial community structure on specific dates. Using Nonmetric Multidimensional Scaling (NMDS) in R's Vegan package, plots were created to visualize beta diversity across treatments, with 95% confidence ellipses added using R's ggplot package. A summary tree showing the UniFrac distances between the average bacterial community structure of each sample type by sampling date combination was created using neighbor joining through FastTree (Price et al., 2009) and visualized using FigTree v1.4.2 (http://tree.bio.ed.ac.uk/software/figtree/).

Alpha (within sample) diversity was assessed in QIIME by Faith's phylogenetic diversity (PD) metric (Faith, 1992), calculated using Fast UniFrac (Hamady et al., 2010). Alpha rarefaction curves showing OTU count by sampling depth were generated by repeatedly (10 iterations), randomly sampling subsets of OTU tables in increasing number from 10

sequences to the specified maximum sampling depth. To compare alpha diversity between groups of samples, 2-sample *t*-tests were conducted using 999 permutations per comparison in a Monte Carlo approach with a Bonferroni correction. Alpha diversity analyses were performed at sampling depths equal to those used for beta diversity comparisons.

2.4 Weather data

Daily precipitation measurements were obtained from the National Oceanic and Atmospheric Administration website (www.noaa.gov), using climatological data collected at the Damascus, Maryland weather station, located 9 km from the sample site. Only limited weather data was available from the Damascus station, so additional weather measurements, including temperature, barometric pressure, and wind speed, were acquired from the Montgomery County Airport Automated Weather Observing Station in Gaithersburg, Maryland. The weather station is operated by the Federal Aviation Administration, and administered by NOAA (National Centers for Environmental Information, Asheville, NC), and data was accessed through Weather Underground (www.wunderground.com).

3. Results

3.1 Sequencing metrics

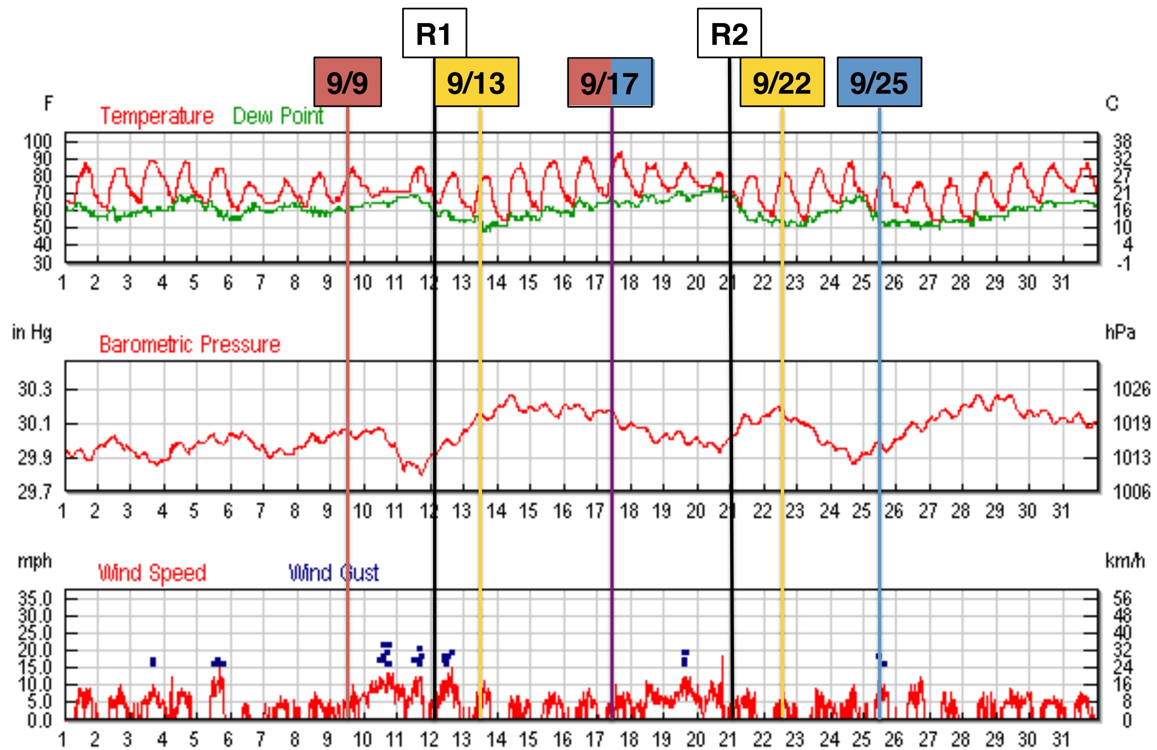
Sequencing on MiSeq v3 resulted in 3.7 million reads from the 94 multiplexed samples included in this study. Reads averaged a length of 463 bases, with an average Q score of 35 and an average of 46,605 reads per sample (±15,136). Reads 1 and 2 were merged at an average efficiency of 83%. For the unmerged reads, read 1 was included for further

analysis. Quality filtering for low quality and non-target sequences removed ~6% of the dataset, leaving 3.6 million reads for downstream analysis.

3.2 Weather

Before the study began, limited rainfall (~1 mm) had fallen since a 21 mm rain event on 8/20, 3 weeks prior to Rain 1. The first rain event (Rain 1) resulted in 9.14 mm of precipitation, and the second event (Rain 2) reached 9.65 mm. Fluctuations in temperature, barometric pressure and wind speed were also noted (Figure 1). The highest daily temperature was recorded on 9/9, and a wind gust occurred 9/13 around sampling time. Barometric pressure was low on 9/9 and 9/13 compared to the other sampling dates. Rain 1 was accompanied by thunder and lightning.

Figure 1. September 2015 weather data from Montgomery County Airport in Gaithersburg, MD, located 71 km from the study site. Base image was provided by Weather Underground (www.wunderground.com/history/), with source data from NOAA's Global Surface Hourly database (National Centers for Environmental Information, Asheville, NC). Dates of sampling (month/date) and Rain 1 (R1) and Rain 2 (R2) are marked.



3.3 Cucumber carpoplane

On the cucumber carpoplane, alpha diversity increased significantly following Rain 1, as measured by both Faith's Phylogenetic Diversity (PD) (p=0.033) and observed OTUs (p=0.030), surging from an average of 1,293 (±306) to 1,743 (±138) OTUs (Operational Taxonomic Units with 97% sequence similarity) per sample, a 34.8% increase (Figure 2A and B). Four days after Rain 1, alpha diversity remained elevated compared to pre-rain levels (p=0.024 and p=0.030 for PD and observed species, respectively), with an average of 1,766 (±281) OTUs per cucumber.

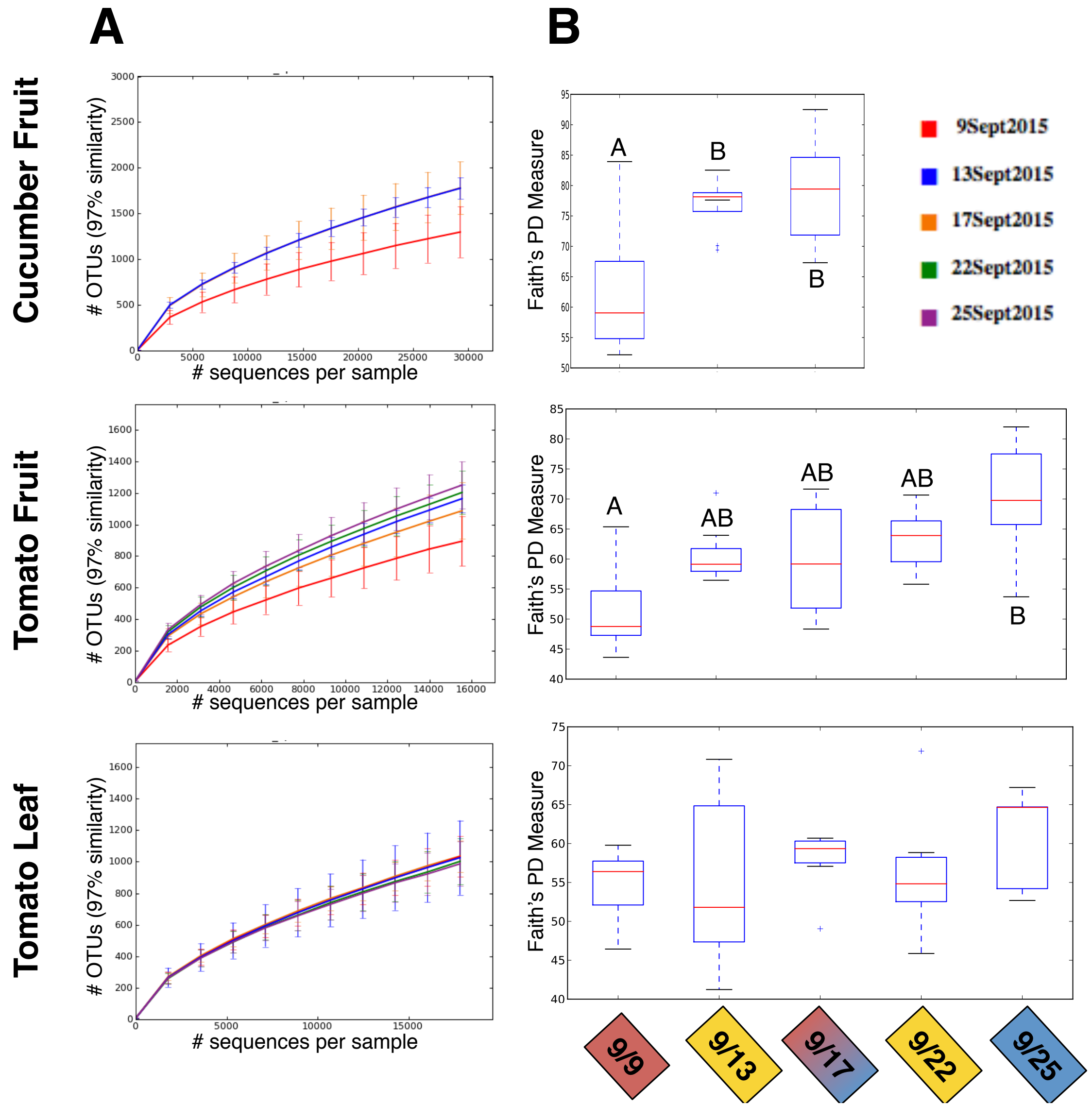


Figure 2: Alpha diversity across sampling dates for cucumber fruit and tomato fruit and leaves. In panel A, the number of OTUs classified at the 97% identity level for each sampling date are marked at 10 intervals approaching the maximum sampling depth, with the variation between 10 iterations marked with error bars. In panel B, box and whisker plots show Faith's Phylogenic Diversity metric for each sampling date, with the median marked as a horizontal line, the box encompassing the interquartile range, and the whiskers showing range excluding outliers. Different letters denote significantly different

(Bonferroni-corrected $p<0.05$ from a 2-sample *t*-test) PD measures, as between sampling dates within each sample type.

In addition to an increase in the bacterial OTU count on cucumber fruit following rain, a significant change in bacterial community structure was observed for both weighted (R^2=0.251, p=0.001) and unweighted (R^2=0.140, p=0.001) analyses (Figure 3A-B). Cucumber fruit samples collected 4 days prior to Rain 1 supported bacterial communities distinct from those identified 1 day after Rain 1 ($p<0.001$, Bonferroni-corrected from 2-sample *t*-test). However, although alpha diversity remained elevated 4 days after Rain 1, beta diversity largely returned to pre-rain community structure. Samples collected 4 days after Rain 1 hosted communities indistinguishable from pre-rain (p=1) and 1-day post-rain (p=0.150) samples (Figure 3A-B).

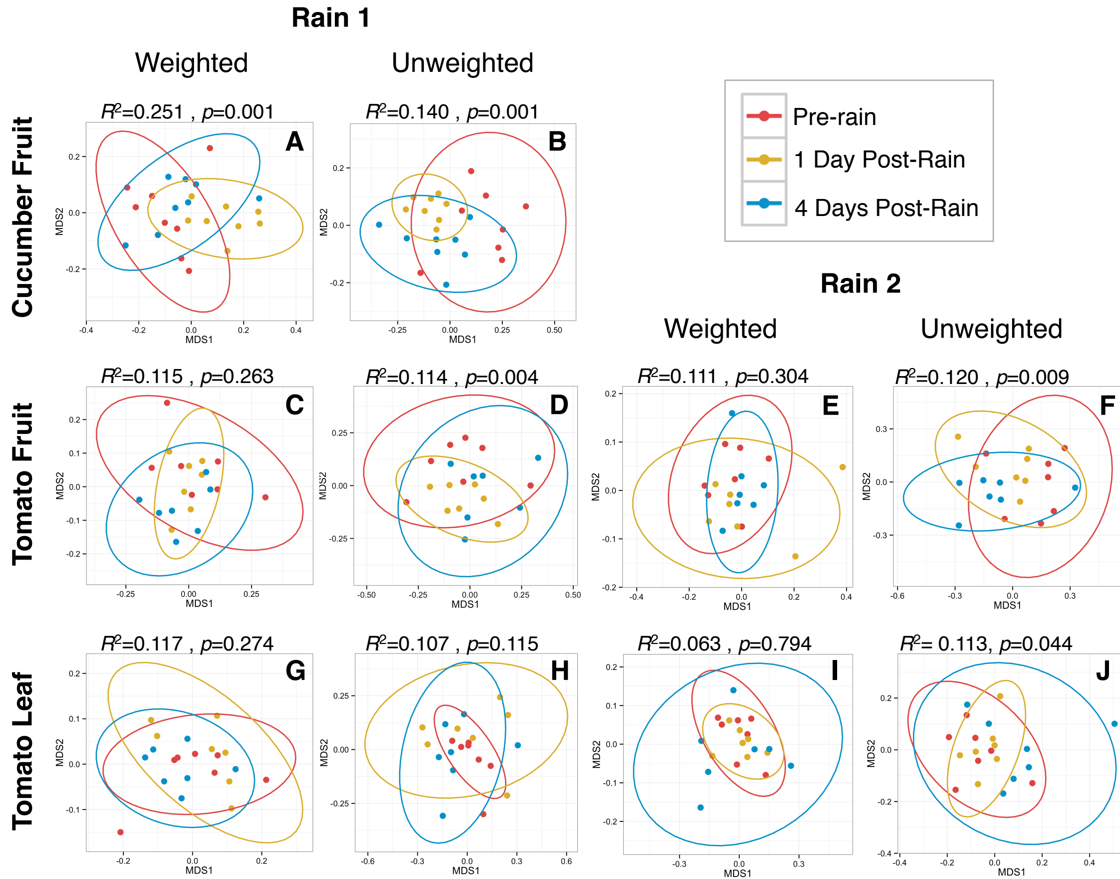


Figure 3: NMDS plots reveal influence of rain on cucumber and tomato-associated bacterial community structure. Non-Metric Multidimensional Scaling was applied to unweighted (including taxal identification only) and weighted (including taxal abundance) UniFrac distance matrices to compare bacterial community structure before, 1 day after, and 4 days after two rain events. One sampling date, 9/17/15, is represented in Rain 1 as the 4 Day Post-rain sample group and in Rain 2 as the Pre-rain sample group. R^2 values, generated through 999 iterations of Adonis, a non-parametric MANOVA, represent the percent variation explained by differences in sampling date for Rain 1 and Rain 2.

Forty-three genera not observed in pre-rain cucumber carpoplane samples were identified in samples collected 1 day post-rain (Figure 4, Supplementary Table 1). Of these, 28 genera were detected 4 days post-rain as well. By contrast, 35 genera observed in pre-rain samples were not detected 1 day post-rain, but 19 of these were observed again 4 days post-rain. All of the potentially introduced or eliminated genera were present at less than 0.01% relative abundance.

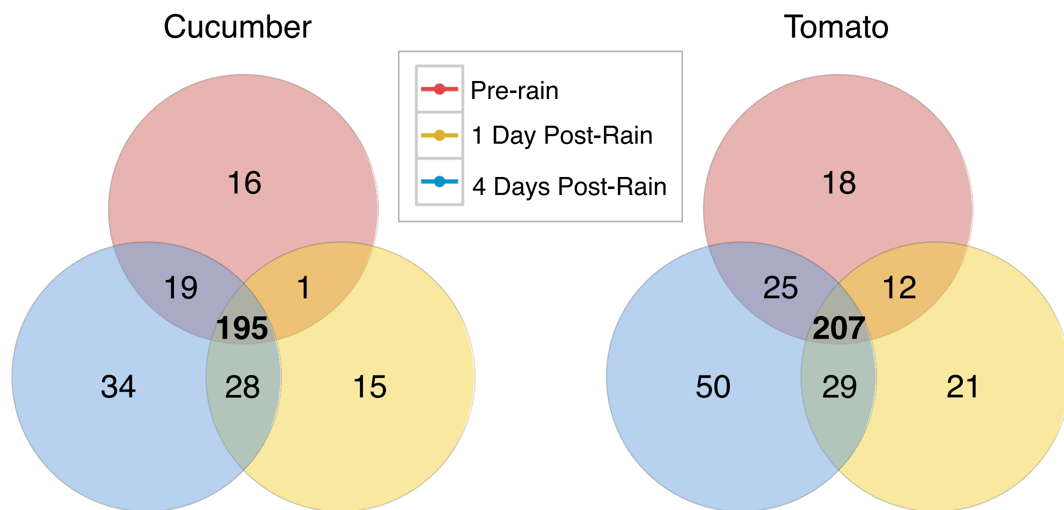


Figure 4. Bacterial genera associated with cucumber and tomato carpoplanes on 3 dates surrounding Rain 1. Genera present on 1, 2, or all 3 dates from 9/9 to 9/17 were tallied and represented in a Venn diagram. The list of ephemeral (present on only 1 or 2 dates) genera summarized here can be found in Supplementary Tables 1 and 2.

In addition to the introduction of new taxa, changes in the abundance of established taxa were observed following Rain 1 (Figure 5). Xanthamonadaceae increased from 0.7% to 5.8% relative abundance following rain, dropping to 1.5% 4 days after rain (FDR-

corrected *p*=0.041). Oxalobacteriaceae experienced a similar increase, from 0.6% to 6.5%, but in this case abundance remained high after 4 days, at 5.4% relative abundance (FDR-corrected *p*=0.011). Similarly, Paenibacilliaceae and Comamonadaceae, initially present at less than 0.1% relative abundance, at least quadrupled in relative abundance following rainfall, remaining elevated 4 days later (FDR-corrected *p*=0.041 and 0.011, respectively). Relative abundances for several of the most dominant bacterial families on the cucumber surface declined or increased following rainfall, though these differences were not statistically significant. Sphingomonadaceae decreased from 20% to 9% relative abundance following rainfall, but recovered 4 days later to 14%. Similarly, the abundance of the Microbacteriaceae diminished following rainfall, resurging to even higher levels later. Enterobacteriaceae demonstrated an opposite shift, increasing 1 day after rain from 13% to 16%, later returning to 12% relative abundance (Figure 5).

At the genus level, *Enterobacter* was significantly different across sampling dates, growing in relative abundance from 0.002% to 0.05% 1 day post-rain and then reducing to 0.03% 4 days post-rain (FDR-corrected *p*=0.035). *Acidovorax*, a genus including the causative agent of bacterial fruit blotch on cucurbits, was not detected in pre-rain samples but was detected at low levels 1 day (0.01%) and 4 days (0.006%) post-rain (FDR-corrected *p*=0.035).

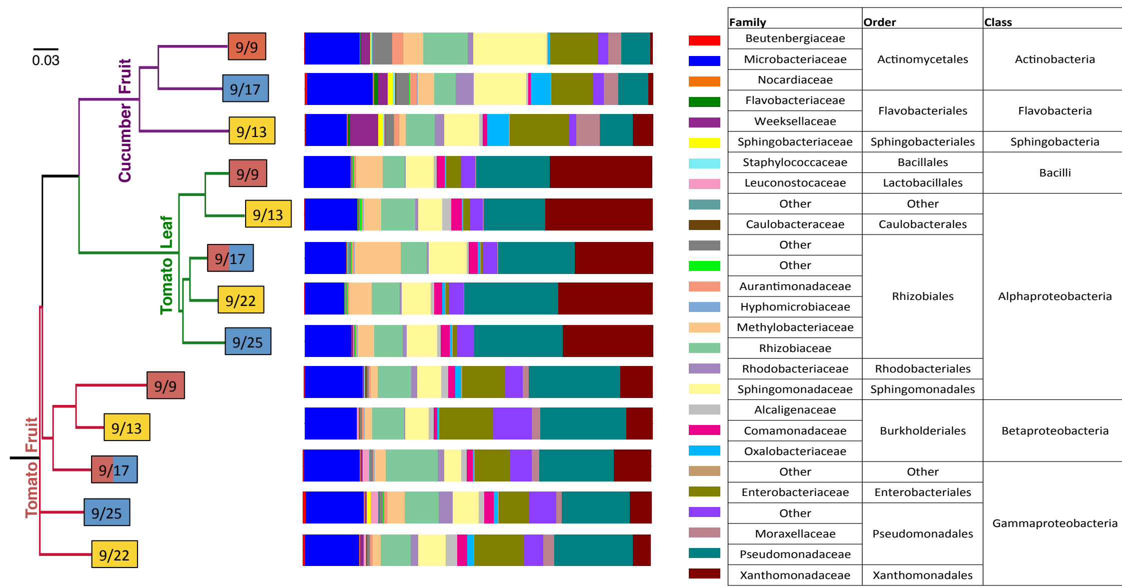


Figure 5: Phylogenetic relatedness and family-level taxonomic profiles of all sample types across the study period. A summary tree, including all taxa present at more than 0.1% relative abundance, was created for each sampling date from the average taxonomic profiles of cucumber fruit (n=8), tomato leaf (n=7), and tomato fruit (n=7) surface washes. The tree was created using the neighbor joining method from a weighted UniFrac distance matrix in QIIME v1.8 and visualized in FigTree v1.4.2. Summarized taxonomic profiles created by QIIME v1.8 are shown at the tips of the trees, also excluding taxa present at less than 0.1% relative abundance.

3.4 Tomato carpoplane

Following Rain 1, alpha diversity on the tomato carpoplane resembled the dynamics seen on cucumbers. Observed OTU count increased from 885 (±148) to 1,161 (±84) OTUs per sample (p=0.050) from 9/9 to 9/13, however this increase was not statistically supported (p=0.200) when phylogenetic relatedness and abundance metrics were included using the PD measure (Figures 2C and D). Four days after Rain 1, alpha diversity by both measures

was indistinguishable from pre-rain and 1 day post-rain levels (p>0.05), at 1,079 (±177) OTUs per sample. Following Rain 2, observed OTU count remained elevated compared to pre-Rain 1 (p=0.03 and p=0.02 for 9/22 and 9/25, respectively) but did not significantly increase beyond 9/17 levels. Analysis of the same dates by PD suggested that across the sampling period, alpha diversity was statistically significantly different only between 9/9 and 9/25 (p=0.04).

Beta diversity of the tomato carpoplane did not necessarily follow the same trend observed on cucumber fruit. There was a weak overall effect of sampling date on bacterial community structure (R^2=0.172, p=0.028). Pairwise comparisons reveal that tomato fruit samples collected on the first sampling date 9/9, 1 day prior to Rain 1, hosted communities that differed in structure only from those collected on 9/22 (p=0.025), 1 day after Rain 2, and 9/25 (p=0.041), 4 days after Rain 2. When tomato fruit data were analyzed separately for Rain 1 and Rain 2, weighted comparisons revealed no differences between samples collected before, immediately after, and 4 days following rain. However, when NMDS was applied to unweighted distance matrices on composition data only, a weak effect was observed for both rain events (Figures 3C-F), with pre-rain and 4 day post-rain samples differing significantly for Rain 2 (p=0.009).

At the genus and family level, several low abundance (<1%) taxa differed in relative abundance across sampling date, but fluctuations in relative abundance did not appear to be related to rain event proximity. At the family level, relative abundance of Burkholderiaceae, Xanthobacteriaceae, and Beijerinkiaceae were significantly different

across sampling dates (FDR-adjusted p=0.025 for all). Relative abundance of Burkholderiaceae and Beijerinkiaceae steadily increased (0.0003% to 0.005% for Burkholderiaceae, 0.001% to 0.01% for Beijerinkiaceae) over the course of the sampling period. Relative abundance of Xanthobacteriaceae increased over the first 3 dates but decreased for the last 2 dates. For the 3 dates surrounding Rain 1, tomato fruits collected on each day supported unique genera not observed on any other date, with 9/17 hosting the most unique genera (Figure 4, Supplementary Table 2).

3.5 Tomato phylloplane

On tomato leaf surfaces, both alpha and beta diversity remained largely consistent regardless of proximity to rainfall, with an average of 982 OTUs per sample (Figures 2E and F). Across the full sampling period, date collected did not significantly influence bacterial community structure at a rarefaction level of 17,375 sequences per sample (R^2=0.124, p=0.384), however unweighted NMDS analysis of Rain 2 data suggests a difference between tomato phylloplane bacterial community structure collected pre-rain and 4 days post-rain (R^2=0.113, p=0.044) (Figure 3J). No taxa shifted in relative abundance throughout the sampling period, however 4 days following Rain 2, tomato leaf sample communities were more varied compared to those collected either before (p=0.001) or immediately after (p=0.004) Rain 2 (Figure 3I-J). The UniFrac distance between averaged sample groups from each collection date suggest a weak shift in community structure over the course of the sampling period. UniFrac distance of samples collected on subsequent dates from 9/9 increased modestly but steadily (Figure 5), however this was not statistically supported by Adonis.

3.6 Cucumber versus tomato fruit

The 2 fruit species studied hosted comparable numbers of bacterial OTUs, supporting an average of 1,118 (±196) OTUs on tomato and 1,208 (±255) on cucumber fruit (p=0.278 for observed OTUs, p=0.851 for PD). Bacterial community structure, however, differed significantly between the 2 fruit types (R^2=0.282, p=0.001). Several of the most dominant taxa differed in relative abundance between cucumber and tomato fruit surfaces, including Psuedomonadaceae, Xanthomonadaceae, Sphingomonadaceae, and Moraxellaceae (FDR-adjusted p<0.001 for all). Tomato fruit were dominated by members of the Pseudomonadaceae (23.8%) and Microbacteriaceae (15.4%), while cucumbers supported high levels of Microbacteriaceae (14.6%), Enterobacteriaceae (14.0%), and Sphingomonadaceae (14.6%) (Figure 5). Cucumber carpoplane bacterial community evenness was higher compared to tomato carpoplane communities ; the five most abundant taxa on tomato fruit surfaces made up 72.3% of total bacterial diversity, compared to 61.2% on cucumber.

4. Discussion and Conclusions

In this study, phytobiome analysis of cucumber and tomato plant surfaces using 16S rRNA gene amplicon sequencing allowed us to assess the impact of rainfall on epiphytic bacterial communities at the time of crop harvest. This analysis demonstrated an increase in bacterial species diversity on cucumber and tomato fruit surfaces following rain events, potentially accompanied by shifting bacterial community dynamics. On the cucumber and tomato carpoplanes, several new bacterial species were introduced following rainfall, maintaining populations with low abundance in the days following rain. On cucumber fruit surfaces, several of the most dominant taxa increased or

decreased in relative abundance following rainfall, often fully or partially returning to pre-rain levels within 4 days. Furthermore, overall bacterial community structure on cucumber fruits shifted significantly in response to rain, when measured both when incorporating phylogenetic diversity of bacteria present (unweighted UniFrac) and when assessing only the relative abundance and relatedness of bacterial OTUs (weighted UniFrac). On the other hand, despite the observed sustained increase in diversity, bacterial community profiles of the tomato fruit surface were statistically similar before and immediately following rain events for comparisons including both phylogenetic relationships and relative abundance of taxa. Tomato fruit samples collected 1 and 4 days after the second rain event supported bacterial communities distinct from the first pre-rain sampling date. An additive effect considering multiple rain events could explain these differences, however other environmental or developmental factors could have played a role as well. Unlike fruit surface communities, tomato leaf surface community structure and diversity remained largely consistent across all sampling dates. A weak shift in community structure was observed in the phylloplane over the course of sample period, similar to that seen on tomato fruits, however no significant differences were detected. The diminished effect on leaves could have been due to the variation in texture on leaves compared to fruits, resulting in heightened protection from rain and rain-associated environmental factors.

Compared to results seen on tomato, microbiota living on the cucumber carpoplane seemed to be more responsive to weather-related changes. Exposure to the elements could explain this effect. Cucumbers were grown on plasticulture on the ground, while

tomatoes were staked upright; while tomato fruit were somewhat shielded by foliage, cucumber fruit were much less protected. Cucumber fruit lying directly on plastic mulch were left both more exposed to direct rainwater contact and closer to the soil, increasing the potential for splash. Newly introduced species may have originated from rain or transferred via splash or wind from soil or nearby plant parts. While the sampling dates were selected to surround rain events, other weather dynamics during the sampling period could not be controlled for and likely influenced phytobiomes as well. Differences in barometric pressure and wind speed (Figure 1) or reduced UV stress due to cloud cover could have influenced phytobiome dynamics or interacted with the factor of rain. Furthermore, rainfall may have been correlated with larger scale ecosystem changes. For example, insect visitation may have been limited during the rain event but elevated in the days following precipitation.

Pesticides were applied to tomato plots during the sampling period, on the evenings of 9/9 and 9/16. It is possible that these applications could have influenced microbiome structure and diversity, however phyllosphere bacterial communities tend to be fairly robust in the face of pesticide application (Ottesen et al., 2015; Perazzolli et al., 2014). Furthermore, although pesticides were not applied to the cucumber plot, similar response to rainfall was observed on cucumbers.

Another factor to consider is that prior to Rain 1, the area experienced a long drought, with negligible rainfall since the last major rain event (21 mm) 3 weeks before the study began. The increase in diversity observed following Rain, 1 but not Rain 2, could be

explained by drought-induced suppression of bacterial diversity at the start of sampling, not replicated prior to Rain 2, which occurred only 9 days later. Due to the close proximity of the rain events, it is possible that bioaerosols were less prevalent during the second rain. Plants release microbes into the atmosphere preferentially on sunny, dry days (Lindemann and Upper, 1985), and there were few of those between the two rain events. Unfortunately, cucumber data for Rain 2 was not collected due to seasonality and a lack of availability of high quality samples, so we cannot address whether this difference between R1 and R2 would have been mirrored on cucumber. The shifts in bacterial OTU richness and in some cases community structure that we did observe following rain events could have been the result of direct inoculation by rainwater-associated microbiota or by other factors associated with rainfall. Rain could physically remove microbes from the plant surface, opening up a niche for others to fill. Alternatively, increased moisture and relative humidity in the air before, during, and after rain events could favor rapid growth of certain taxa at the expense of others. Changes in other factors, such as decreased UV exposure and altered animal visitation could contribute to the growth and establishment of bacterial populations on the plant surface as well.

While it is important to understand the local influence of isolated rainfall events on microbial dynamics in agriculture, in the future it will also be important to consider the influence of weather patterns on a larger scale. In addition to the direct impact of rainfall on phytobiomes, prolonged wet or dry periods could influence plant immune response, and storms could lead to wounding, creating opportunities for pathogens to infiltrate plant

tissues. Climatic changes could lead to expanded ranges for plant pathogens due to favorable wet and warm conditions in higher latitudes and changing dispersal patterns influenced by intensified rain and wind and shifting vector habitats (Sutherst et al., 2011). Many factors associated with climate change, including elevated temperatures, increased periods of drought, intensified storms, and elevated CO_2 will likely influence the health of our crops directly (Pautasso et al., 2010), but also indirectly by shifting the balance of microbes that may inhabit them, including plant pathogens, human pathogens, and beneficial or non-detrimental organisms. For some plant-pathogen pairs, weather-based forecasting models are already in use, helping growers time pesticide applications efficiently for the highest effectiveness and lowest environmental impact (Bakeer et al., 2013; Isard et al., 2015; Shtienberg, 2013). Similar decision support systems could be implemented for use in food safety, providing recommendations to growers on safest harvest times following single and repeated rain events. The first step in achieving this goal is amassing an understanding of the community-level dynamics on harvestable fresh produce preceding and following rain events.

Chapter 5: Insect exclusion limits variation in tomato blossom and fruit microbiomes

1. Introduction

The phyllosphere, characterized by aboveground plant surfaces including leaves, blossoms, and fruit, is a dynamic environment supporting diverse microbiota including bacteria, fungi, and archaea (Aleklett et al., 2014; Delmotte et al., 2009; Lindow and Brandl, 2003; Maignien et al., 2014; Rastogi et al., 2013; Vorholt, 2012). In agriculture, fruits represent a large share of consumable specialty crops, and blossoms are intimately connected to the development of those crops. Both blossoms and fruit support active microbial communities, with bacteria as some of the most dominant constituents (Bulgarelli et al., 2013; Vorholt, 2012). However there is limited research into the dynamics of bacterial communities on blossoms and fruit and the driving forces that shape the structure of these communities (Leff and Fierer, 2013; Perazzolli et al., 2014; Shade et al., 2013; Zarraonaindia et al., 2015). The phyllosphere microbiome may be influenced by a variety of factors including environmental conditions, host genotype, plant developmental stage, and plant organ (Bodenhausen et al., 2014; Bulgarelli et al., 2013; Micallef et al., 2009a; Ottesen et al., 2013; Rastogi et al., 2012). Various plant organs (blossoms, leaves, and fruit) have consistently been shown to host distinct bacterial communities on the same plant (Junker et al., 2011; Ottesen et al., 2013).

The tomato blossom habitat offers protection from phyllosphere stressors such as UV and provides relatively high humidity and consistent nutrient availability due to the presence of nectar and pollen (Aleklett et al., 2014). Tomato fruit are comparatively more exposed

to environmental stresses but release plant surface compounds, such as sugars, that may support specific communities of microbes. In fact, different cultivar-specific exudate profiles influenced the survival of *Salmonella enterica* on tomato fruit (Han and Micallef, 2016), and grape, plum and nectarine exudates have been shown to influence *Botrytis cinerea* growth (Fourie et al., 1998; Padgett and Morrison, 1990). This physiological variation may explain some of the differences between phyllosphere microbiota found on blossom and fruit surfaces, but differences in the types of insects that visit these plant organs may play a role as well. Insect visitors, whether they be pollinators or pests, likely host distinct microbiomes, both in their guts and on their body surfaces. There is strong evidence that microbes may be transmitted to plant organs through insect feeding (Caspi-Fluger et al., 2012; Sugio et al., 2015), excretion (Mitchell and Hanks, 2009; Soto-Arias et al., 2014), or body contact (Martinson et al., 2012; Ushio et al., 2015).

Recent studies have shown that insects can transmit bacteria, including human and plant pathogens, to flowers. Pollinators can carry over a million microbial cells on their surfaces, and during flower visitation leave a microbial fingerprint (Aizenberg-Gershtein et al., 2013; de Vega and Herrera, 2013; Ushio et al., 2015). Both naturally- and artificially- infected flies can act as hosts and vectors for foodborne pathogens including *Cronobacter*, *Salmonella enterica*, and *Listeria monocytogenes* (Holt et al., 2007; Olsen, 1998; Pava-Ripoll et al., 2015). After transmission to the flower, these pathogens may internalize into fruit and persist as part of the internal fruit microbiome (Guo et al., 2001; Zheng et al., 2013). Herbivorous insects, more often found on leaves, can transmit phytopathogenic or commensal bacteria and fungi through feeding, potentially acting as

both host and vector (Nadarasah and Stavrinides, 2011). Moreover, droppings from phytophageous insects feeding on nearby leaves could come into contact with developing fruit, serving as a potential source of microbial inoculum.

More research is needed to characterize the importance of insect visitation on blossom and fruit surfaces for fruit crops commonly consumed raw. In this study, we investigated the influence of insect exclusion on bacterial microbiota associated with field-grown tomato blossom and developing fruit surfaces. We used 16S rRNA gene sequencing to assess variation in bacterial community diversity and structure and examine the dynamics of specific bacterial taxa on fruit and blossoms collected from plants completely covered in netting, to exclude insects, or exposed to the environment throughout blossom and fruit development.

2. Materials and Methods

2.1 Field setup

This study was conducted concurrently with the soil amendment study (Chapter 3). Four plants, located in rows 3 and 4 (Supplementary Figure 1), were covered in nylon mosquito netting when plants began to blossom. Before installing netting, all blossoms and developing fruit were removed to ensure that during subsequent sampling only blossoms and fruit produced under netted conditions would be collected. Netting was adjusted throughout the season to allow for plant growth. Non-netted plants were selected for bacterial community profiling from the same plot containing netted plants.

2.2 Sample collection and processing

Blossom and green fruit samples were collected and processed in August 2014, as detailed in Chapter 3. Briefly, blossom and green fruit were collected aseptically into Ziploc bags and transported back to the lab on ice, where surface wash microbes were collected onto 0.22 micron filters (Nalgene Nunc International Corporation, Rochester, NY). DNA extractions were performed using MoBio's Powerwater kit (MoBio, Carlsbad, CA), and the V1-V3 portion of the 16S rRNA gene was amplified using Illumina's library preparation guidelines (Illumina, Part#15044223 rev. B). Amplicons were multiplexed with samples from Chapter 3 and run on the Illumina MiSeq v3 platform.

2.3 Microbial community analysis

Quality filtering of sequences and bacterial community structure and diversity analyses were performed as described in Chapter 3. NMDS plots were created in R and statistical analyses were conducted using weighted UniFrac distance matrices produced through QIIME v1.8.

3. Results

3.1 From blossom to green fruit

Green fruit supported significantly more phylogenetic diversity compared to blossoms, as measured by both Operational Taxonomic Unit (OTU) richness (p=0.001) and Faith's Phylogenetic Diversity measure (p=0.004) (Figure 1). Blossom and green fruit hosted distinct epiphytic bacterial communities, both in terms of overall bacterial community structure (R^2=0.27, p=0.001, Adonis nonparametric PERMANOVA) (Figure 2A) and in terms of specific taxonomic differences (Appendix 1 Table 3). One of the most dominant phyllosphere families, Xanthomonadaceae, differed significantly in relative abundance

between blossom and fruit surfaces, constituting 35% of bacteria identified on blossom and only 14% on fruit (FDR-p=0.038). At the order level, green fruit supported more members of Rhizobiales compared to blossoms (FDR-p=0.029). Most of this difference could be explained by a high relative abundance (20%) of bacteria classified as *Agrobacterium* on fruit surfaces; relative abundance of this organism was only 6% on blossoms (FDR-p=0.054). Several other highly abundant taxa differed in abundance between blossom and fruit surfaces, however these differences were not significant (Appendix 1 Table 3).

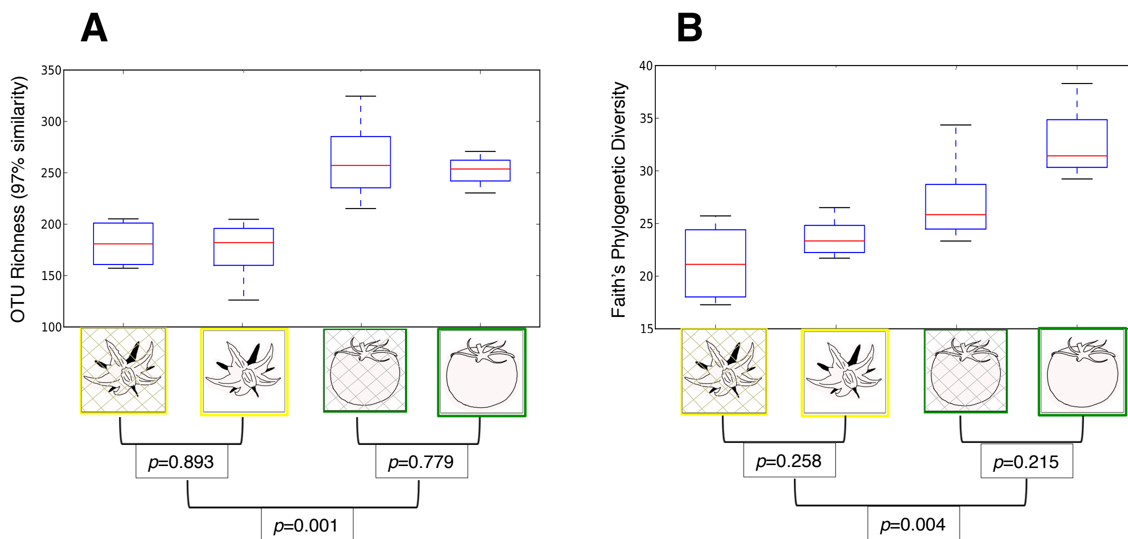
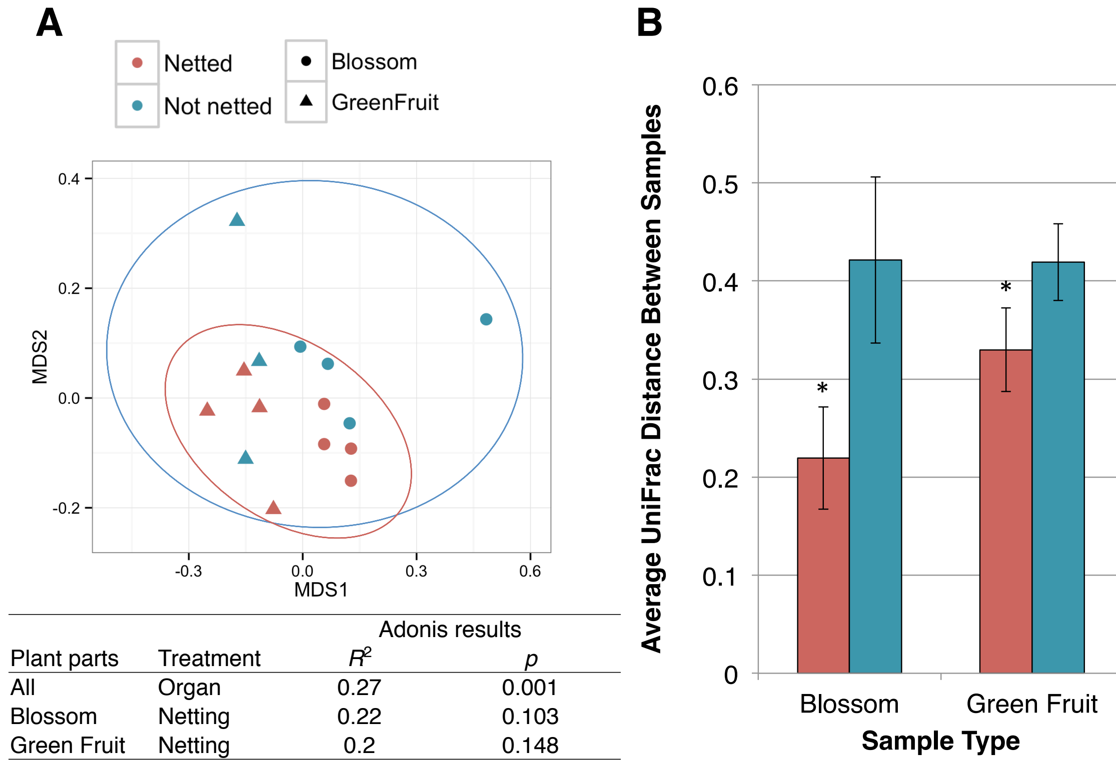


Figure 1. Alpha diversity of bacterial communities collected from insect-excluded or control tomato blossom and green fruit surfaces. Panel A shows the number of operational taxonomic units identified at 97% sequence identity at a depth of 1,210 sequences per sample for each sample type. Panel B displays Faith's PD measure at the same sampling depth. The median for each set of samples is marked as a horizontal red line, the box encompasses the interquartile range, and the whiskers show range excluding

outliers. Bonferroni-corrected *p* values calculated from two-sample *t*-tests for each comparison (netted vs. non-netted for each sample type, blossom vs. green fruit regardless of sample type) are shown below the graphs.



Plant parts	Treatment	Adonis results R^2	p
All	Organ	0.27	0.001
Blossom	Netting	0.22	0.103
Green Fruit	Netting	0.2	0.148

Figure 2. Community structure and within-treatment heterogeneity. Nonmetric Multidimensional Scaling (NMDS) was applied to a weighted UniFrac distance matrix to visualize clustering of samples by treatment, and Adonis nonparametric MANOVA was applied to assess significance between treatments (Panel A). The average phylogenetic distance between netted and non-netted samples of each sample type is shown in Panel B (with standard deviations), as measured from the weighted UniFrac distance matrix. Significance (Bonferroni-corrected $p<0.05$ reached by 2-sided 2-sample *t*-test) is shown for each sample type with an asterisk.

3.2 Insect exclusion did not significantly alter taxonomic richness in the phyllosphere

Within sample (alpha) diversity was assessed through direct counts of OTUs at 97% sequence identity and through Faith's Phylogenetic Diversity measure (PD), which incorporates the phylogenetic difference between OTUs (Faith, 1992). While OTU richness did not differ between netted and non-netted samples for both blossom (p=0.893) and green fruit (p=0.779) (Figure 1A), the inclusion of phylogenetic relatedness in the analysis with the use of Faith's PD measure revealed some distinction between netted and non-netted samples. Non-netted samples supported higher average PD, although the differences were not statistically significant for either blossoms (p=0.258) or fruit (p=0.215) (Figure 1B).

3.3 Insect exclusion limits variation between blossom and fruit microbiomes

Netted blossom surfaces did exhibit some differences in bacterial communities compared to non-netted blossoms (R^2=0.22, p=0.103, Adonis nonparametric PERMANOVA). Differences were most marked in the spread of samples collected from netted and non-netted plants (Figure 2A). Blossoms shielded from insect visitation via netting supported much more consistent community structure compared to those left exposed. Average similarity in community structure among netted blossom samples was double that of non-netted samples, as measured by average UniFrac distance between sample pairs (p=0.004) (Figure 2B). Similar trends were observed on green tomato fruit as those seen on blossoms. No statistically significant influence of netting was detected for bacterial community structure on green fruit surfaces (R^2=0.20, p=0.148, Adonis nonparametric PERMANOVA). However, fruit samples collected from non-netted plants supported

bacterial communities with approximately 10% higher dissimilarity than those from netted plants (p=0.038) (Figure 2B).

Construction of a phylogenetic tree through neighbor joining revealed that blossom samples hosted bacterial communities that were more phylogenetically similar to each other than to those on non-netted blossoms, however netted and non-netted fruit did not consistently follow this trend (Figure 3).

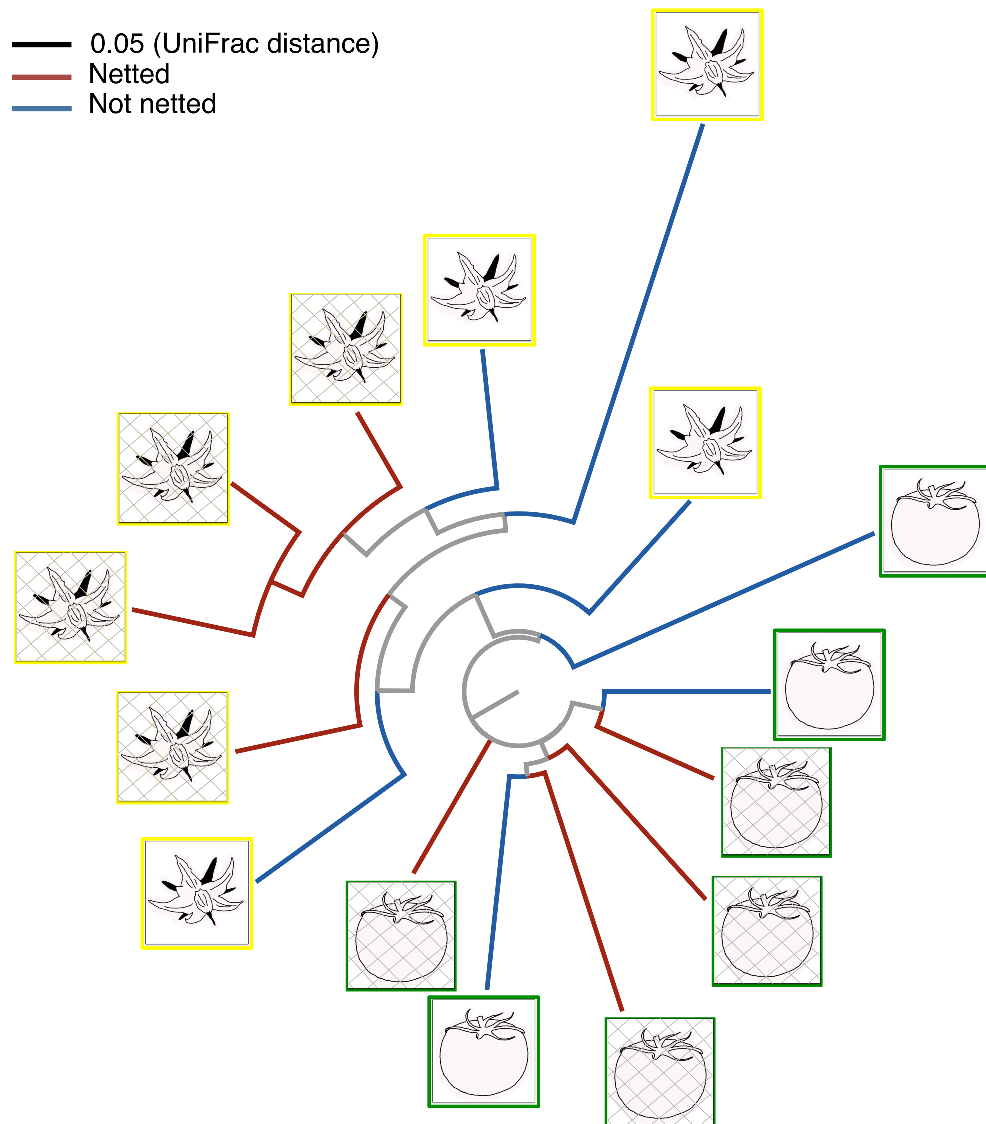


Figure 3. Neighbor joining tree showing phylogenetic relatedness of netted and non-netted blossom and fruit surface microbiomes. All OTUs (97% identity) present at more than 0.1% relative abundance were included in construction of the tree, which was created using the neighbor joining method from a weighted UniFrac distance matrix in QIIME v1.8 and visualized in FigTree v1.4.2. Blossom samples are represented in yellow boxes and green fruit samples in green boxes, with netting treatment marked with a boxed overlay. Tree branches are colored by netting treatment.

3.4 Relative abundances of specific bacteria on netted and non-netted phyllosphere samples

Non-netted blossom surfaces supported consistently higher, consistently lower, or more variable abundances compared to netted blossoms for several taxa (Figure 4, Appendix 1 Table 4). On netted blossoms, the relative abundance of the Pseudomonadaceae was consistent, between 8.7% and 10.7%. By contrast, non-netted blossom surfaces supported more variable Pseudomonadaceae abundances, ranging from 0.6% to 15.1% (Figure 5). Non-netted blossoms also supported elevated relative abundances of Sphingobacteriaceae and Shewanellaceae. Non-netted fruit displayed similar patterns, supporting higher populations of Bacillaceae and Methylobacteriaceae, and less variable levels of Rhizobiaceae, compared to their netted counterparts. Enterobacteriaceae, a bacterial family including several important foodborne and plant pathogens, was consistently elevated on non-netted blossom surfaces, ranging from 3.2% to 6.5% relative abundance compared to 1.6%-3.4% on netted samples. This effect was not mirrored on green fruit surfaces, where Enterobacteriaceae levels varied widely across both netted and not netted samples, ranging from 0.9% to 34.3% relative abundance (Figure 5).

On non-netted blossoms, often one sample displayed an elevated relative abundance in a certain taxa compared to the rest of the samples. For the four non-netted blossom samples, Microbacteriaceae, Rhizobiaceae, Psuedomonadaceae, and Xanthamonadaceae were highly elevated in relative abundance for samples 1, 2, 3, and 4, respectively but lower and fairly consistent for the other 3 samples (Figure 5). For netted blossoms, screened from insect visitation, no such extreme variations were observed. On fruit, both non-netted and netted samples had relatively high variation between samples of the same treatment group, however non-netted fruit samples did show slightly more variation in relative abundance for several taxa. Individual non-netted samples supported greatly decreased Microbacteriaceae (sample 4), elevated Sphingobacteriaceae (sample 1), and elevated Comamonadaceae (sample 4) and well as the sample with the highest relative abundance of Enterobacteriaceae (sample 2), at 34.3% (Figure 5). Non-netted fruit sample 3 had elevated Pseudomonadaceae, however the netted fruit sample from the same plot had similarly high abundance of this taxa, as did the non-netted blossom sample from the same plot (Figure 5).

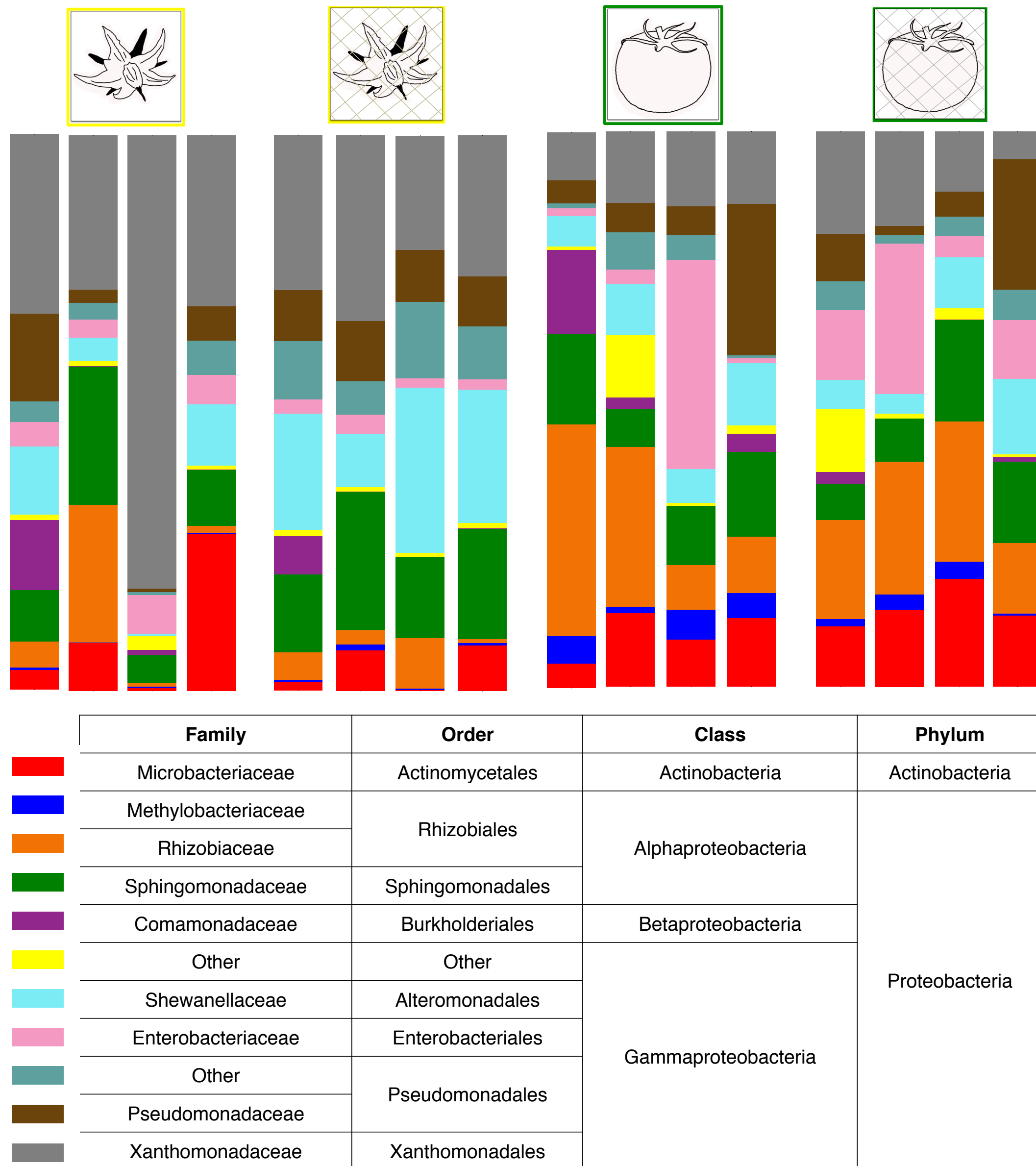


Figure 4. Family-level bacterial community profiles of netted and non-netted blossom and fruit samples. Families with relative abundance >1% were scaled up to 100% to visually represent the family-level diversity of all sample types. The full taxonomy of represented families is listed in the legend.

Taxonomy				Fruit samples: Netted				Fruit samples: Not netted			
Phylum	**Class**	**Order**	**Family**	1	2	3	4	1	2	3	4
Actinobacteria	Acintobacteria	Actinomycetales	Microbacteriaceae	13.53%	17.01%	11.94%	10.72%	10.92%	7.76%	11.79%	3.88%
Bacteriodetes	Flavobacteria	Flavobacteriales	Weeksellaceae	0.15%	0.02%	0.00%	0.01%	0.07%	0.07%	0.00%	3.39%
	Sphingobacteria	Sphingobacteriales	Sphingobacteriaceae	0.40%	2.56%	0.01%	0.00%	5.20%	0.07%	0.00%	0.83%
Firmicutes	Bacilli	Bacillales	Bacillaceae	0.05%	0.27%	0.01%	0.12%	1.93%	0.07%	0.29%	0.83%
			Paenibacillaceae	0.01%	0.07%	5.99%	0.18%	1.15%	0.07%	0.15%	0.17%
Proteobacteria	Alphaproteobacteria	Rhizobiales	Methylobacteriaceae	2.68%	2.71%	0.26%	1.28%	0.95%	4.95%	4.22%	4.29%
			Rhizobiaceae	23.34%	22.01%	11.87%	17.42%	23.62%	7.25%	9.61%	33.36%
			Phyllobacteriaceae	0.13%	0.00%	0.05%	0.37%	0.01%	0.00%	0.24%	1.45%
		Rhodobacterales	Rhodobacteraceae	0.14%	0.16%	0.00%	0.00%	1.01%	0.07%	0.00%	0.83%
	Betaproteobacteria	Burkholderiales	Alcaligenaceae	0.00%	2.27%	0.00%	0.04%	1.12%	3.80%	0.00%	1.90%
			Comamonadaceae	0.01%	0.11%	0.78%	2.18%	1.66%	0.10%	3.06%	13.13%
			Oxalobacteraceae	0.07%	3.31%	0.07%	0.15%	0.00%	0.75%	0.15%	0.25%
	Gammaproteobacteria	Alteromonadales	Shewanellaceae	3.02%	1.47%	4.98%	5.02%	5.54%	4.07%	0.44%	0.74%
		Enterobacteriales	Enterobacteriaceae	26.42%	3.33%	9.84%	12.37%	2.10%	34.34%	0.87%	1.24%
		Pseudomonadales	Pseudomonadaceae	1.65%	3.99%	21.81%	8.42%	4.29%	4.68%	25.91%	3.63%
		Xanthomonadales	Xanthomonadaceae	16.54%	9.40%	4.64%	18.08%	10.58%	12.31%	12.37%	7.51%

Taxonomy				Blossom samples: Netted				Blossom samples: Not netted			
Phylum	**Class**	**Order**	**Family**	1	2	3	4	1	2	3	4
Actinobacteria	Acintobacteria	Actinomycetales	Microbacteriaceae	8.01%	7.22%	0.15%	1.51%	27.90%	8.39%	3.34%	0.47%
Bacteriodetes	Flavobacteria	Flavobacteriales	Weeksellaceae	0.00%	0.00%	0.00%	0.00%	0.01%	0.00%	0.02%	0.00%
	Sphingobacteria	Sphingobacteriales	Sphingobacteriaceae	0.00%	0.00%	0.00%	0.00%	0.00%	0.11%	0.10%	0.03%
Firmicutes	Bacilli	Bacillales	Bacillaceae	0.00%	0.00%	0.03%	0.01%	0.03%	0.22%	0.02%	0.06%
			Paenibacillaceae	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Proteobacteria	Alphaproteobacteria	Rhizobiales	Methylobacteriaceae	0.40%	1.06%	0.27%	0.34%	0.17%	0.05%	0.42%	0.28%
			Rhizobiaceae	0.67%	2.52%	8.87%	4.73%	1.28%	24.03%	4.52%	0.54%
			Phyllobacteriaceae	0.14%	0.37%	0.04%	0.01%	5.64%	0.07%	0.15%	0.17%
		Rhodobacterales	Rhodobacteraceae	0.00%	0.00%	0.00%	0.00%	0.00%	0.05%	0.03%	0.00%
	Betaproteobacteria	Burkholderiales	Alcaligenaceae	0.01%	0.00%	0.00%	0.00%	0.00%	0.00%	0.96%	0.03%
			Comamonadaceae	0.05%	0.12%	0.03%	6.60%	0.14%	0.11%	12.07%	0.88%
			Oxalobacteraceae	0.08%	0.00%	0.09%	1.17%	0.04%	0.00%	0.04%	1.33%
	Gammaproteobacteria	Alteromonadales	Shewanellaceae	9.39%	23.42%	20.00%	28.94%	10.91%	4.00%	11.77%	0.38%
		Enterobacteriales	Enterobacteriaceae	1.81%	3.40%	1.63%	2.53%	5.23%	3.24%	4.17%	6.54%
		Pseudomonadales	Pseudomonadaceae	8.72%	10.62%	9.20%	8.81%	6.12%	2.30%	15.08%	0.57%
		Xanthomonadales	Xanthomonadaceae	24.69%	32.92%	20.05%	26.69%	30.30%	26.88%	30.80%	75.87%

Figure 5. Relative abundance of select bacterial taxa on netted and non-netted blossom and green fruit surfaces. Families with >0.1% relative abundance in at least one sample type were examined and selected if they exhibited variation between netted and non-netted samples. Conditional formatting was applied in Excel to color highest abundances for each taxon in dark green and less abundant taxa along a gradient toward yellow. The gradient is unique to each row to best highlight differences between netted and non-netted samples within each sample type for each family shown. Columns marked with the same numbers denote samples collected from the same plots. Average relative abundances are shown for each sample for all taxa.

4. Discussion and Conclusions

Enhanced knowledge of the relationships between insects, the plants they visit, and the microbiota they may transmit will increase our understanding of what factors influence microbial assemblages associating with crops and, consequently, plant health and food safety. With this study, we investigated the influence of insect exclusion on the bacterial microbiota associated with field-grown tomato blossom and fruit surfaces. We found that blossom and green fruit hosted unique bacterial communities, and these communities responded to restricted insect visitation at different scales.

Field-grown green tomato fruit supported significantly higher taxonomic diversity and distinct bacterial community structure compared to blossoms grown on the same plants, mimicking differences seen between blossoms and red fruits (Ottesen et al., 2013). Although blossoms provide a protected and nutritious habitat for microorganisms, they also can release secondary metabolites that may limit microbial success, effectively preserving nectar and pollen for pollinators (Pozo et al., 2012). This antimicrobial effect could account for the difference observed in operational taxonomic richness between blossom and fruit surfaces.

Insect exclusion from tomato plants had a more dramatic effect on blossom-associated bacterial communities compared to green fruit-associated communities. Blossoms collected from the 4 plants covered in mosquito netting shared very similar bacterial profiles, while the 4 plants exposed to insect visitation supported a more diverse array of bacterial community profiles. Notably, non-netted blossoms supported consistently

higher relative abundances of the Enterobacteriaceae, a family including several enteric pathogens of concern for human health. Indeed, studies have shown that insects can host and transmit members of this family, such as *Salmonella enterica* and *Cronobacter*, and foodborne pathogens from other families, such as *Listeria monocytogenes* of Listeraceae (Holt et al., 2007; Olsen, 1998; Pava-Ripoll et al., 2015). The blossom is a potential point of entry for foodborne pathogens to reach fruit, the marketable portion of tomato plants. Due to the documented potential of foodborne pathogen internalization from blossom to fruit (Zheng et al., 2013), the environmental transmission of Enterobacteriaceae by insects may be of concern for food safety.

Insects have been confirmed as vehicles for microbial inoculation to flowers, not only for individual taxa but for diverse assemblages of microbes (Ushio et al., 2015). Honeybees, important pollinators of many economically important agricultural crops, may support microbial community structures indistinguishable from the flowers that they visit (Aizenberg-Gershtein et al., 2013), and wild bees similarly acquire bacteria from flowers (McFrederick et al., 2012). On grapefruit and sweet almond trees, netted and non-netted flowers supported significantly different bacterial communities, with non-netted flowers and honeybees sharing similar community structure (Aizenberg-Gershtein et al., 2013). The less dramatic effect observed in our study could be explained by the structure of the tomato flower. Tomato flowers have the potential to self-fertilize, and reproductive structures are more shielded compared to other flowers with more easily accessible floral rewards. Unlike flowers that host more specialist pollinators, tomato flowers are visited by a wide variety of generalist insects. Exposure to diverse flower habitats could lead to a

diversity of microbial profiles later transmitted to the tomato flower upon visitation. Although the environment does seem to be a source for bee-associated microbiota (McFrederick et al., 2014), bee microbiome structure may not be correlated with visitation to certain floral habitats (Aizenberg-Gershtein et al., 2013; McFrederick and Rehan, 2016). Moreover, this study examined the blossom as an entire entity, but it would be interesting to investigate whether the introduction of non-core taxa is specific to particular floral components, which can host unique microbial fingerprints (Andrews and Harris, 2000; Junker and Keller, 2015; Pozo et al., 2012). Nectar in particular has been studied extensively and contains a diverse array of fungal and bacterial species (Alvarez-Pérez et al., 2012; Pozo et al., 2012) that can differ in community structure by plant species (Fridman et al., 2012).

Green fruit are less likely to attract insect visitors compared to blossoms due to their tough, waxy exterior and lack of easily accessible food source for insects. If insects did visit the fruit, they would likely leave visible feeding damage, reducing marketability of fruit. In this study, we only harvested marketable fruit, so none of the samples collected here were visibly afflicted with insect feeding. Any differences in non-netted versus netted bacterial profiles on fruit would likely be the result of blossom-associated differences persisting through fertilization and fruit development, or differences in contact with herbivorous insect appendages, or insects releasing droppings onto fruit. In general, we found that bacterial communities on fruit surfaces were similar between netted and non-netted plants. Similar to blossoms, netted fruit samples were significantly more similar to each other than non-netted fruit were to each other, however this effect

was less pronounced on fruit. Relative abundance of many taxa varied widely between non-netted fruit samples, leading to between-sample variation in bacterial community structure comparable to that of non-netted blossoms. Variation in netted fruits, however, was slightly higher than variation observed between netted blossom samples, so the difference between netted and non-netted fruit sample variation was diminished. While the introduction of taxa through insect visitation may be important in establishing diverse blossom microbiomes throughout fields, this seems less important for fruits, although the effect cannot be disentangled from carryover in microbial constituents from blossom to fruit.

This study revealed an insect exclusion effect on tomato-associated bacterial communities, an effect that was stronger on blossoms but partially conveyed to the green fruit surface. A study with more replication over space and time may be able to tease out some of the dynamics and clarify the relationships between insect visitors and phyllosphere bacterial structure.

Chapter 6: Conclusions and future directions

In today's world, agriculture faces formidable challenges. Global climate change may lead to increased dispersal of pathogens, plant stress, and crop loss (Scholthof, 2007). The world population continues to grow, as does world hunger and the demand for fresh produce. Foodborne disease outbreaks linked to fresh produce are common worldwide. With enhanced understanding of plant-associated microbial community dynamics, manipulation of microbial communities to maximize plant growth, defense, and nutrient uptake efficiency will be possible, allowing more efficient use of limited resources and reduced side effects of agricultural inputs. Recent advances in genomic technology have made investigations into the diversity and community structure of microbial communities possible in many systems and at multiple scales, creating exciting opportunities to increase our understanding of microbial dynamics in agriculture.

In this study, plant genotype and plant organ were the strongest drivers of overall bacterial community structure in the specialty crop phyllosphere and rhizosphere despite exposure to diverse nutritional and environmental treatments. Environmental factors did influence plant-associated microbiomes, as was seen with field location in Chapter 3, rainfall in Chapter 4, and insect exclusion in Chapter 5. However these factors never caused differences in bacterial community structure greater than those between blossoms, fruit, and roots, or between tomato and cucumber fruits. Although the influences of these factors were somewhat subtle, they should not be discounted.

Although the same methods and even in some cases the same fields were used for the 3 studies, the scale and character of microbial changes were unique for each objective, as were the specific analyses required to tease out these changes. In the soil amendments study, the application of microbiologically diverse soil amendments did not result in any measurable change in bacterial communities associated with tomato roots, blossoms, and fruit. Instead, existing soil texture and water activity gradients were more influential on bacterial community structure than the agricultural management practice investigated. When assessing the influence of rain, one of the most striking effects observed was the increase in taxonomic richness following the first rainfall, observed for both cucumber and tomato fruits. For both fruit types, distance matrices considering all taxa at equal abundances detected a stronger rainfall effect compared to those with OTUs weighted by abundance, suggesting that the additional taxa identified following rain were large in number but not abundant enough to cause significant community-level shifts. Still, many of these taxa were retained in the days following rainfall, showing that taxa introduced following rainfall can persist in the carposphere. In Chapter 5, overall bacterial community structures were statistically similar between blossom and green fruit samples collected from plants grown under insect exclusion or exposed to insect visitation. However, an investigation into the spread of diversity between treatments revealed that netted blossom and fruit-surface bacterial communities were highly similar to each other, while non-netted samples supported a wider array of bacterial profiles. This again suggests that certain environmental exposures can lead to sustained changes in plant microbiomes. These changes may occur at a small scale, but the influence of the rare biosphere may be proportionally larger than specific bacterial abundances suggest, and

given supportive conditions these low abundance microbial populations could grow at any time (Elshahed et al., 2008; Lynch and Neufeld, 2015; Pedrós-Alió, 2007). Furthermore, if plant or human enteric pathogens were to be part established through these environmental routes, even small abundances could have a large impact on human and plant health (Brandl, 2006).

Just as pharmaceutical companies are investigating the power of microbes as alternative medicine to treat human disease (Reardon, 2014), interest in manipulating plant-associated microbial communities is growing in terms of agricultural biotechnology (Berg, 2009). Microbial dynamics of the rhizosphere have been studied much more extensively compared to those of the phyllosphere, and as a result rhizosphere research has moved forward more substantially from description to application in the field. Physical, chemical, and biological characteristics of soils have been investigated in connection to their influence on rhizosphere community structure and diversity across many systems (Bossio et al., 1998; Liu et al., 2007; Termorshuizen et al., 2006; Wu et al., 2008). This robust characterization has led to a greater understanding of how microbes can be used to enhance agricultural production and environmental sustainability (Bakker et al., 2012; Berg, 2009; Chaparro et al., 2012). Further research into the basic dynamics of the phyllosphere in response to varied agricultural practices will lead to similar advances in practical application. Some potential uses of microorganisms to enhance agricultural productivity include biological control, disease suppressive soils, and plant growth promotion.

Bacterial and fungal biocontrols are becoming an important part of agricultural management, and demand for them is steadily increasing (Berg, 2009). The natural tendency of some microbes to enhance plant response to abiotic and biotic stresses may be utilized by isolating and applying these beneficial microbes in higher-than-endemic concentrations in the field. Several microbes have been investigated as potential biocontrols for foodborne pathogen infection (Allard et al., 2014), however biocontrols used to control plant pathogens are much more widely studied. Microbial community characterization using –omics technologies represent a new avenue for discovery of potential biocontrol microorganisms. In addition to specific strains of microorganisms with antimicrobial activity, microbiome analysis could help characterize the effectiveness of assemblages of microbes with demonstrated biocontrol characteristics, such as disease-suppressive soils. Several biological, chemical, and physical soil characteristics are correlated with disease suppressiveness (Liu et al., 2007; Termorshuizen et al., 2006); in the future predictive models may be able to forecast compatibility and effectiveness with different systems based on these characteristics as well as microbiome profiles. Due to the complexity and specificity of plant-pathogen-environment interactions, use of disease suppressive soils will be most effective if tailored specifically based on host, pathogen, and environment. With these concerns addressed, disease suppressive composts and biological control agents have potential as environmentally friendly, safe, and effective approaches to disease control. The added benefits associated with biocontrols and disease suppressive soil application may also be explored; in addition to disease suppression these often support enhanced nutrient uptake by host plants (Berg, 2009; Harman, 2000).

Continuing efforts to assess the balance between plants, their associated microorganisms, and the environment will lead to a richer understanding of the potentially far-reaching influences of crop management choices and environmental conditions on agricultural productivity and food safety. As we continue to characterize environmental and human-driven factors in crop production that may lead to large- and small-scale microbial changes with consequences for agricultural production, we will move toward innovations in produce safety, plant pathogen management, and sustainability. Microorganisms are a vast and diverse force on Earth, intimately connected to our own bodies, the food we eat, the homes we live in, and virtually all of our planet's processes. As we work toward enhanced food safety and security and strive toward environmental sustainability, it is crucial that we not only consider how our actions may influence our microbial neighbors, but also how we can harness their power to realize our vision for a better world.

Appendix 1: Supplementary tables

Table 1: Low abundance taxa absent from cucumber fruit samples collected on 1 or 2 dates surrounding Rain 1

Present only 9/9 (4 days pre-Rain 1)				
Phylum	**Class**	**Order**	**Family**	**Genus**
Actinobacteria	Actinobacteria	Actinomycetales	Kineosporiaceae	Unclassified
				Unclassified
			Micrococccaceae	Microbispora
			Thermomonosporaceae	Unclassified
Firmicutes	Bacilli	Bacillales	Listeraceae	Listeria
		Lactobacillales	Leuconostocaceae	Unclassified
	Clostridia	Clostridiales	[Tissierellaceae]	Unclassified
Proteobacteria	Alphaproteobacteria	Rhizobiales	Methylocystaceae	Unclassified
		Rickettsiales	mitochondria	Unclassified
	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	Xenorhabdus
		Oceanospirillales	Halomonadaceae	Candidatus Portiera
		Oceanospirallales	Unclassified	Unclassified
		Thiotrichales	Piscirickettsiaceae	Unclassified
		Xanthamonadales	Xanthamonadaceae	Luteibacter
		Xanthamonadales	Xanthamonadaceae	Xanthomonas
		Vibrionales	Vibrionaceae	Vibrio

Present only 9/13 (1 day post-Rain 1)				
Phylum	**Class**	**Order**	**Family**	**Genus**
Actinobacteria	Actinobacteria	Actinomycetales	Gordoniaceae	Millisia
Bacteriodetes	Cytophagia	Cytophagales	Cytophagaceae	Rhodocytophaga
Cyanobacteria	Oscillatoriophycideae	Oscillatoriales	Phormidiaceae	Phormidium
	Synechococcophycideae	Pseudanbaenales	Pseudanabaenaceae	Leptolygbya
Firmicutes	Bacilli	Bacillales	Bacillaceae	Geobacillus
		Lactobacillales	Enterococcaceae	Unclassified
	Clostridia	Clostridiales	Lachnospiraceae	Unclassified
			Ruminococcaceae	Unclassified
Proteobacteria	Alphaproteobacteria	Rickettsiales	mitochondira	Trebouxia
		Rhizobiales	Rhizobiaceae	Rhizobium
	Betaproteobacteria	Burkholderiales	Alcaligenaceae	Kerstersia
			Burkholderiaceae	Unclassified
	Gammaproteobacteria	Xanthomonadales	Unclassified	Unclassified
		Pseudomonadales	Moraxellaceae	Alkanindiges
Chloroflexi	Anaerolineae	SBR1031	A4b	Unclassified

Present only 9/17 (4 days post-Rain 1)				
Phylum	**Class**	**Order**	**Family**	**Genus**
Actinobacteria	Actinobacteria	Actinomycetales	Actinosynnemataceae	Unclassified
			Dietziaceae	Dietzia
			Frankiaceae	Frankia
			Jonesiaceae	Jonesia
			Propionibacteriaceae	Propionibacterium
			ACK-M1	Unclassified
			Nocardioidaceae	Propionicimonas
			Propionibacteriaceae	Unclassified
	Thermoleophilia	Solirubrobacterales	Unclassified	Unclassified
Bacteriodetes	[Saprospirae]	[Saprospirales]	Chitinophagaceae	Niabella
	Cytophagia	Cytophagales	Cytophagaceae	Adhaeribacter
Firmicutes	Bacilli	Bacillales	Unclassified	Unclassified
			Paenibacillaceae	Aneurinibacillus
			Planococcaceae	Planococcus

Phylum	Class	Order	Family	Genus
			Thermoactinomycetaceae	Unclassified
		Lactobacillales	Lactobacillaceae	Unclassified
			Unclassified	Unclassified
	Clostridia	Clostridiales	Eubacteriaceae	Pseudoramibacter_Eubacterium
Proteobacteria	Alphaproteobacteria	Rhizobiales	Methylocystaceae	Pleomorphomonas
			Phyllobacteriaceae	Nitratireductor
		Rhodospirillales	Rhodospirillaceae	Unclassified
	Betaproteobacteria	Unclassified	Unclassified	Unclassified
		Burkholderiales	Comamonadaceae	Limnohabitans
			Oxalobacteraceae	Massilia
	Deltaproteobacteria	Bdellovibrionales	Bdellovibrionaceae	Bdellovibrio
		Myxococcales	Unclassified	Unclassified
			Myxococcaceae	Unclassified
	Gammaproteobacteria	Alteromonadales	Alteromonadaceae	Marinimicrobium
			HTCC2188	HTCC
		Enterobacteriales	Enterobacteriaceae	Buchnera
		Legionellales	Unclassified	Unclassified
			Coxiellaceae	Unclassified
		Pasteurellales	Unclassified	Unclassified
Chloroflexi	Thermomicrobia	JG30-KF-CM45	Unclassified	Unclassified

Present 9/13 and 9/17 only (1 and 4 days post-Rain 1)				
Phylum	**Class**	**Order**	**Family**	**Genus**
Actinobacteria	Actinobacteria	Actinomycetales	Intrasporangiaceae	Phycicoccus
			Micrococcaceae	Unclassified
			Nocardiaceae	Nocardia
			Nocardioidaceae	Pimelobacter
			Pseudonocardiaceae	Pseudonocardia
			Streptosporangiaceae	Nonomuraea
	Thermoleophilia	Solirubrobacterales	Unclassified	Unclassified
			Solirubrobacteraceae	Unclassified
	Unclassified	Unclassified	Unclassified	Unclassified
Firmicutes	Bacilli	Bacillales	Alicyclobacillaceae	Alicyclobacillus
			Planococcaceae	Planomicrobium
				Unclassified
		Lactobacillales	Carnobacteriaceae	Desemzia
			Streptococcaceae	Unclassified
Proteobacteria	Alphaproteobacteria	Unclassified	Unclassified	Unclassified
		Rhizobiales	Bradyrhizobiaceae	Bosea
			Methylocystaceae	Unclassified
		Rhodospirillales	Acetobacteraceae	Belnapia
	Betaproteobacteria	Burkholderiales	Burkholderiaceae	Unclassified
			Oxalobacteraceae	Telluria
		MKC10	Unclassified	Unclassified
		Rhodocyclales	Rhodocyclaceae	Unclassified
	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	Serratia
				Trabulsiella
		Oceanospirillales	Endozoicimonaceae	Unclassified
	Unclassified	Unclassified	Unclassified	Unclassified
TM7	Unclassified	Unclassified	Unclassified	Unclassified
[Thermi]	Deinococci	Deinococcales	Trueperaceae	Truepera

Present 9/9 and 9/17 only (4 days pre- and 4 days post-Rain 1)				
Phylum	**Class**	**Order**	**Family**	**Genus**
Actinobacteria	Actinobacteria	Actinomycetales	Brevibacteriaceae	Brevibacterium
			Geodermatophilaceae	Unclassified
			Gordoniaceae	Unclassified
			Propionibacteriaceae	Unclassified
	Thermoleophilia	Gaiellales	Gaiellaceae	Unclassified
Bacteroidetes	Flavobacteriia	Flavobacteriales	[Weeksellaceae]	Unclassified

Cyanobacteria	Chloroplast	Chlorophyta	Unclassified	Unclassified
Firmicutes	Bacilli	Bacillales	Planococcaceae	Lysinibacillus
		Lactobacillales	Enterococcaceae	Unclassified
Planctomycetes	Planctomycetia	Gemmatales	Gemmataceae	Gemmata
Proteobacteria	Alphaproteobacteria	Caulobacterales	Caulobacteraceae	Phenylobacterium
		Rhizobiales	Bradyrhizobiaceae	Unclassified
		Rhodospirillales	Rhodospirillaceae	Unclassified
		Sphingomonadales	Unclassified	Unclassified
	Betaproteobacteria	Neisseriales	Neisseriaceae	Unclassified
	Deltaproteobacteria	Myxococcales	0319-6G20	Unclassified
	Gammaproteobacteria	Xanthomonadales	Sinobacteraceae	Unclassified
TM6	SJA-4	Unclassified	Unclassified	Unclassified
[Thermi]	Deinococci	Deinococcales	Deinococcaceae	Deinococcus

Present 9/9 and 9/13 only (4 days pre- and 1 day post-Rain 1)				
Phylum	**Class**	**Order**	**Family**	**Genus**
Proteobacteria	Alphaproteobacteria	Rickettsiales	Rickettsiaceae	Wolbachia

Table 2: Low abundance taxa absent from tomato fruit samples collected on 1 or 2 dates surrounding Rain 1

Present only 9/9 (4 days pre-Rain 1)				
Phylum	**Class**	**Order**	**Family**	**Genus**
Actinobacteria	Actinobacteria	Actinomycetales	Intrasporangiaceae	Knoellia
				Phycicoccus
			Micromonosporaceae	Virgisporangium
			Nocardioidaceae	Pimelobacter
			Thermomonosporaceae	Unclassified
Firmicutes	Bacilli	Lactobacillales	Leuconostocaceae	Unclassified
	Clostridia	Clostridiales	Peptostreptococcaceae	Tepidibacter
Planctomycetes	Planctomycetia	Gemmatales	Gemmataceae	Gemmata
Proteobacteria	Alphaproteobacteria	Rhizobiales	Beijerinckiaceae	Unclassified
			Methylocystaceae	Pleomorphomonas
		Rhodobacterales	Rhodobacteraceae	Rubellimicrobium
	Betaproteobacteria	Neisseriales	Neisseriaceae	Unclassified
	Deltaproteobacteria	Bdellovibrionales	Bdellovibrionaceae	Bdellovibrio
		Myxococcales	Myxococcaceae	Unclassified
	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	Proteus
		Legionellales	Unclassified	Unclassified
		Xanthomonadales	Sinobacteraceae	Steroidobacter
			Unclassified	Unclassified

Present only 9/13 (1 day post-Rain 1)				
Phylum	**Class**	**Order**	**Family**	**Genus**
Acidobacteria	Acidobacteria-6	CCU21	Unclassified	Unclassified
Actinobacteria	Acidimicrobiia	Acidimicrobiales	EB1017	Unclassified
	Actinobacteria	Actinomycetales	ACK-M1	Unclassified
			Actinosynnemataceae	Unclassified
			Microbacteriaceae	Cryocola
Bacteroidetes	Cytophagia	Cytophagales	Cytophagaceae	Hymenobacter
Chloroflexi	Chloroflexi	AKIW781	Unclassified	Unclassified
	Thermomicrobia	Unclassified	Unclassified	Unclassified
Cyanobacteria	Oscillatoriophycideae	Oscillatoriales	Phormidiaceae	Phormidium
	Synechococcophycideae	Pseudanabaenales	Pseudanabaenaceae	Leptolyngbya
	Unclassified	Unclassified	Unclassified	Unclassified
Proteobacteria	Alphaproteobacteria	Unclassified	Unclassified	Unclassified
		BD7-3	Unclassified	Unclassified
		Rhizobiales	Phyllobacteriaceae	Nitratireductor
	Betaproteobacteria	Burkholderiales	Comamonadaceae	Rhodoferax
			Oxalobacteraceae	Telluria
		Neisseriales	Neisseriaceae	Vitreoscilla
	Deltaproteobacteria	Myxococcales	Haliangiaceae	Unclassified
	Gammaproteobacteria	Aeromonadales	Aeromonadaceae	Unclassified
		Alteromonadales	Alteromonadaceae	Marinimicrobium
		Legionellales	Coxiellaceae	Unclassified

Present only 9/17 (4 days post-Rain 1)				
Phylum	**Class**	**Order**	**Family**	**Genus**
Acidobacteria	[Chloracidobacteria]	PK29	Unclassified	Unclassified
	Acidobacteriia	Acidobacteriales	Koribacteraceae	Unclassified
Actinobacteria	Actinobacteria	Actinomycetales	Bogoriellaceae	Georgenia
			Microbacteriaceae	Agromyces
			Micrococcaceae	Citricoccus
			Micromonosporaceae	Couchioplanes
			Nocardiaceae	Unclassified
			Propionibacteriaceae	Unclassified
			Streptomycetaceae	Unclassified

			Thermomonosporaceae	Actinomadura
	Thermoleophilia	Gaiellales	Gaiellaceae	Unclassified
Bacteroidetes	[Saprospirae]	[Saprospirales]	Chitinophagaceae	Flavisolibacter
				Niabella
	Cytophagia	Cytophagales	[Amoebophilaceae]	Candidatus Cardinium
			Cytophagaceae	Rhodocytophaga
				Spirosoma
	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Myroides
Chloroflexi	Gitt-GS-136	Unclassified	Unclassified	Unclassified
Firmicutes	Bacilli	Bacillales	Alicyclobacillaceae	Alicyclobacillus
			Bacillaceae	Natronobacillus
			Listeriaceae	Listeria
			Paenibacillaceae	Aneurinibacillus
			Planococcaceae	Unclassified
			Thermoactinomycetaceae	Unclassified
		Lactobacillales	Aerococcaceae	Facklamia
				Marinilactibacillus
			Streptococcaceae	Unclassified
	Clostridia	Clostridiales	Unclassified	Unclassified
			Clostridiaceae	Alkaliphilus
				Clostridium
			Lachnospiraceae	Dorea
	Erysipelotrichi	Erysipelotrichales	Erysipelotrichaceae	Erysipelothrix
Gemmatimonadetes	Gemm-1	Unclassified	Unclassified	Unclassified
Planctomycetes	Phycisphaerae	Phycisphaerales	Unclassified	Unclassified
Proteobacteria	Alphaproteobacteria	Caulobacterales	Caulobacteraceae	Mycoplana
		Rhizobiales	Bartonellaceae	Unclassified
			Bradyrhizobiaceae	Afipia
			Phyllobacteriaceae	Phyllobacterium
			Rhizobiaceae	Rhizobium
			Xanthobacteraceae	Azorhizobium
		Rhodobacterales	Rhodobacteraceae	Dinoroseobacter
		Rickettsiales	mitochondria	Trebouxia
	Betaproteobacteria	Burkholderiales	Alcaligenaceae	Kerstersia
	Epsilonproteobacteria	Campylobacterales	Campylobacteraceae	Arcobacter
	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	Xenorhabdus
		Legionellales	Coxiellaceae	Aquicella
		Oceanospirillales	Halomonadaceae	Candidatus Portiera
				Halomonas
		Vibrionales	Pseudoalteromonadaceae	Unclassified
TM7	TM7-3	Unclassified	Unclassified	Unclassified

Present 9/13 and 9/17 only (1 and 4 days post-Rain 1)				
Phylum	**Class**	**Order**	**Family**	**Genus**
Acidobacteria	Solibacteres	Solibacterales	Unclassified	Unclassified
Actinobacteria	Acidimicrobiia	Acidimicrobiales	Unclassified	Unclassified
	Actinobacteria	Actinomycetales	Dermabacteraceae	Unclassified
			Kineosporiaceae	Unclassified
			Micromonosporaceae	Actinoplanes
			Promicromonosporaceae	Unclassified
			Propionibacteriaceae	Propionibacterium
	Thermoleophilia	Solirubrobacterales	Unclassified	Unclassified
			Patulibacteraceae	Patulibacter
		Unclassified	Unclassified	Unclassified
Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae	Unclassified
	Flavobacteriia	Flavobacteriales	[Weeksellaceae]	Unclassified
	Sphingobacteriia	Sphingobacteriale	Sphingobacteriaceae	Unclassified
Cyanobacteria	Unclassified	Unclassified	Unclassified	Unclassified
	Chloroplast	Chlorophyta	Unclassified	Unclassified
Firmicutes	Bacilli	Bacillales	Staphylococcaceae	Jeotgalicoccus

		Turicibacterales	Turicibacteraceae	Turicibacter
	Clostridia	Clostridiales	Clostridiaceae	Unclassified
			Lachnospiraceae	Unclassified
Nitrospirae	Nitrospira	Nitrospirales	Nitrospiraceae	Unclassified
Proteobacteria	Alphaproteobacteria	Rhizobiales	Brucellaceae	Unclassified
			Phyllobacteriaceae	Defluvibacter
		Rhodospirillales	Acetobacteraceae	Acetobacter
				Swaminathania
	Betaproteobacteria	Burkholderiales	Comamonadaceae	Lampropedia
				Ramlibacter
	Deltaproteobacteria	Myxococcales	Polyangiaceae	Unclassified
	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	Serratia
TM7	TM7-3	EW055	Unclassified	Unclassified

Present 9/9 and 9/17 only (4 days pre- and 4 days post-Rain 1)				
Phylum	**Class**	**Order**	**Family**	**Genus**
Unclassified	Unclassified	Unclassified	Unclassified	Unclassified
Actinobacteria	Acidimicrobiia	Acidimicrobiales	C111	Unclassified
	Actinobacteria	Actinomycetales	Cellulomonadaceae	Actinotalea
			Frankiaceae	Unclassified
			Geodermatophilaceae	Geodermatophilus
			Gordoniaceae	Millisia
			Pseudonocardiaceae	Actinomycetospora
				Unclassified
Firmicutes	Bacilli	Bacillales	Bacillaceae	Unclassified
			Planococcaceae	Unclassified
			Unclassified	Unclassified
		Lactobacillales	Unclassified	Unclassified
			Enterococcaceae	Unclassified
	Clostridia	Clostridiales	Ruminococcaceae	Unclassified
				Ruminococcus
Proteobacteria	Alphaproteobacteria	Rhizobiales	Bradyrhizobiaceae	Bosea
		Rhodospirillales	Unclassified	Unclassified
	Betaproteobacteria	Unclassified	Unclassified	Unclassified
	Deltaproteobacteria	Myxococcales	Myxococcaceae	Corallococcus
			Polyangiaceae	Sorangium
	Gammaproteobacteria	Alteromonadales	Alteromonadaceae	Cellvibrio
		Chromatiales	Ectothiorhodospiraceae	Unclassified
		Enterobacteriales	Enterobacteriaceae	Trabulsiella
		Thiotrichales	Piscirickettsiaceae	Unclassified
TM7	TM7-3	Unclassified	Unclassified	Unclassified

Present 9/9 and 9/13 only (4 days pre- and 1 day post-Rain 1)				
Phylum	**Class**	**Order**	**Family**	**Genus**
Acidobacteria	[Chloracidobacteria]	RB41	Ellin6075	Unclassified
Actinobacteria	Acidimicrobiia	Acidimicrobiales	AKIW874	Unclassified
	Actinobacteria	Actinomycetales	Cellulomonadaceae	Unclassified
Bacteroidetes	Cytophagia	Cytophagales	Cytophagaceae	Siphonobacter
Firmicutes	Bacilli	Lactobacillales	Leuconostocaceae	Fructobacillus
Proteobacteria	Alphaproteobacteria	Rhizobiales	Bradyrhizobiaceae	Bradyrhizobium
			Xanthobacteraceae	Xanthobacter
		Sphingomonadales	Erythrobacteraceae	Erythrobacter
	Betaproteobacteria	Burkholderiales	Alcaligenaceae	Pigmentiphaga
		Rhodocyclales	Rhodocyclaceae	Azoarcus
	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	Citrobacter
Verrucomicrobia	Verrucomicrobiae	Verrucomicrobiales	Verrucomicrobiaceae	Luteolibacter

Table 3. Dominant and significantly different taxa between tomato blossoms and fruit

Taxonomic level	Taxon		Mean relative abundance	
		FDR-p	Blossom	Green Fruit
Phylum	Proteobacteria	0.059	92.5%	87.5%
	Actinobacteria	0.059	7.2%	11.8%
	Cyanobacteria	0.038*	0.2%	0.0%
Class	Gammaproteobacteria	0.053	67.8%	47.8%
	Alphaproteobacteria	0.062	22.0%	35.6%
	Actinobacteria	0.064	7.2%	11.8%
	Betaproteobacteria	0.279	2.8%	4.1%
Order	Xanthomonadales	0.020*	34.8%	13.8%
	Rhizobiales	0.029*	7.1%	24.4%
	Sphingomonadales	0.406	14.9%	11.2%
	Pseudomonadales	0.440	14.0%	11.0%
	Alteromonadales	0.406	14.2%	8.6%
	Actinomycetales	0.128	7.2%	11.8%
	Enterobacteriales	0.635	3.7%	11.0%
	Burkholderiales	0.406	2.8%	4.1%
	Unclassified Gammaproteobacteria	0.589	1.0%	3.4%
	Streptophyta	0.046*	0.2%	0.0%
Family	Xanthomonadaceae	0.038*	34.8%	13.8%
	Rhizobiaceae	0.064	5.9%	20.3%
	Sphingomonadaceae	0.399	14.8%	11.1%
	Shewanellaceae	0.399	14.0%	8.5%
	Microbacteriaceae	0.155	7.2%	11.6%
	Pseudomonadaceae	0.756	7.3%	7.4%
	Enterobacteriaceae	0.716	3.7%	11.0%
	Unclassified Pseudomonadales	0.390	6.7%	3.6%
	Comamonadaceae	0.716	2.4%	2.8%
	Unclassified Gammaproteobacteria	0.633	1.0%	3.4%
	Methylobacteriaceae	0.064	0.4%	2.7%
	Alcaligenaceae	0.128	0.1%	1.0%
Genus	Unclassified Xanthomonadaceae	0.026*	31.6%	11.4%
	Unclassified Actinomycetales	0.038*	0.0%	0.2%
	Agrobacterium	0.054	5.9%	20.2%
	Shewanella	0.380	14.0%	8.5%
	Sphingomonas	0.105	13.6%	7.2%
	Microbacterium	0.237	7.0%	9.4%
	Unclassified Enterobacteriaceae	0.701	3.6%	10.1%
	Pseudomonas	0.522	7.0%	5.2%

	Unclassified Pseudomonadales	0.364	6.7%	3.6%
	Unclassified Comamonadaceae	0.483	2.3%	2.6%
	Unclassified Gammaproteobacteria	0.591	1.0%	3.4%
	Luteimonas	0.026*	2.6%	0.4%
	Unclassified Methylobacteriaceae	0.048*	0.4%	2.5%
	Unclassified Sphingomonadaceae	0.083	1.0%	1.9%
	Unclassified Pseudomonadaceae	0.048*	0.2%	2.1%
	Curtobacterium	0.045*	0.2%	2.1%
	Novosphingobium	0.048*	0.1%	1.9%
	Xanthomonas	0.026*	0.2%	0.0%

Table 4. Summary of taxa differing across netted and non-netted blossoms and green fruit

Consistently more relatively abundant on netted samples

Sample	Level	Taxa	Sample	Level	Taxa
Blossom	Family	Pseudomonadaceae	Fruit	Family	Microbacteriaceae
		Unclassified Pseudomonadales		Genus	Curtobacterium
	Genus	Shewanella			Erwinia
		Unclassified Chromatiaceae			

Consistently more relatively abundant on non-netted samples

Sample	Level	Taxa	Sample	Level	Taxa
Blossom	Family	Sphingobacteriaceae	Fruit	Family	Bacillaceae
		Enterobacteriaceae			Methylobacteriaceae
		Shewanellaceae		Genus	Methylobacterium
					Sphingobium
					Bacillus

Non-netted samples vary in relative abundance compared to netted samples

Sample	Level	Taxa	Sample	Level	Taxa
Blossom	Family	Sphingomonadaceae	Fruit	Family	Rhizobiaceae
		Pseudomonadaceae			
	Genus	Pseudomonas		Genus	Unclassified Sphingobacteriaceae
		Sphingomonas			Agrobacterium
		Unclassified Xanthomonadaceae			

1 or 2 non-netted samples have elevated relative abundance compared to other samples

Sample	Level	Taxa	Sample	Level	Taxa
Blossom	Family	Microbacteriaceae	Fruit	Family	[Weeksellaceae]
		Rhizobiales;Other			Sphingobacteriaceae
		Phyllobacteriaceae			Bacillaceae
		Rhizobiaceae			Rhodobacteraceae
		Comamonadaceae			Comamonadaceae
		[Weeksellaceae]			Phyllobacteriaceae
		Alcaligenaceae		Genus	Chryseobacterium
		Xanthomonadaceae			Bacillus
	Genus	Microbacterium			Devosia
		Agrobacterium			Mesorhizobium
		Sphingobium			Kaistobacter
		Bacillus			Novosphingobium
					Achromobacter

Identified on netted samples only

Sample	Level	Taxa
Blossom	Family	Rhodobacteraceae

Identified on non-netted samples only

Sample	Level	Taxa
Blossom	Genus	Sphingobacterium
		Achromobacter

Appendix 2: Field maps

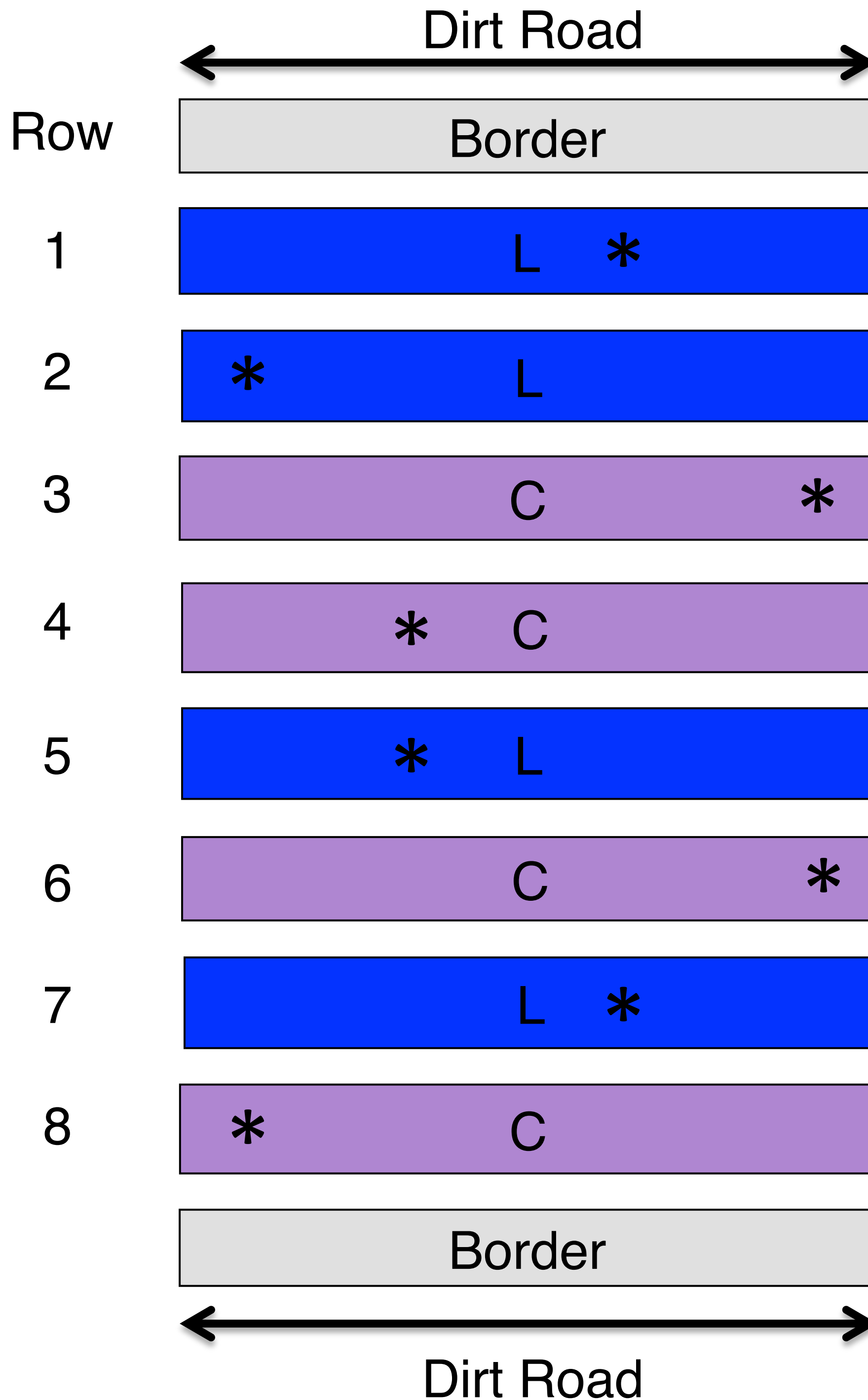


Figure 1. 2013 Field map. Rows spaced 4.6m apart were amended with fresh poultry litter (L) or mineral fertilizer only (C). Asterisks denote approximate sampling location within the rows, ~15m in length and containing ~40 tomato plants each.

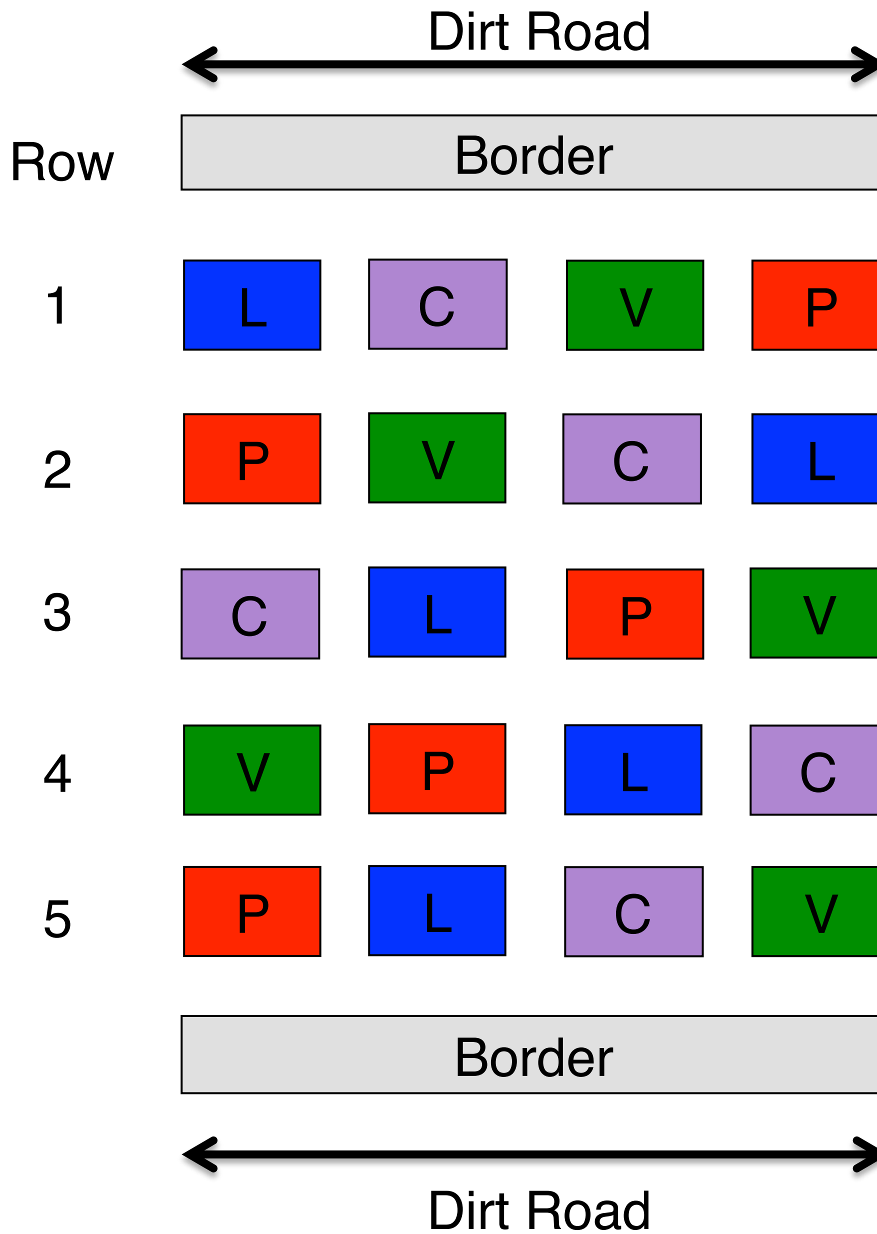


Figure 2. 2014 Field map. Plots spaced 1.5m apart within 15m rows were amended with fresh poultry litter (L), sterile poultry litter pellets (P), vermicompost (V) or mineral fertilizer only (C) and planted with 8 tomato plants each. Rows were spaced 4.6m apart.

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