ABSTRACT

Title of Document: MICROBIOLOGICAL ASSESSMENT OF ORGANIC PRODUCE PRE- AND POST- HARVEST ON MARYLAND FARMS AND IMPACT OF GROWING AND HANDLING METHODS ON EPIPHYTIC BACTERIA

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Although the consumption of organic produce has dramatically increased in recent years and many outbreaks continue to occur, the microbiological safety of organic produce has not been fully assessed. This study generated microbiological data to evaluate organic produce safety and also assessed the impact of growing methods (ground cover effects) and handling methods (washing practices) in organic lettuce systems. The study evaluated microbiological safety of pre- and post-harvest fresh produce samples from small organic farms in Maryland, the effect of mulching on survival of indicator bacteria and the impact of post-harvest washing method on microbiological safety and epiphytic bacterial communities. Results indicate that (1) washed post-harvest produce had higher

risks than unwashed and pre-harvest organic produce as measured by indicator bacteria *E. coli*, total coliforms, APC, yeast and mold; (2) different mulches affected the microbial levels differently; (3) different washing methods altered the bacterial communities both immediately and following 5 days of storage. This study presents data that could be used to assess food safety risks of organic produce associated with their cultivation and on-farm handling practices.

MICROBIOLOGICAL ASSESSMENT OF ORGANIC PRODUCE PRE- AND POST-HARVEST ON MARYLAND FARMS AND IMPACT OF GROWING AND HANDLING METHODS ON EPIPHYTIC BACTERIA

By

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Dedication

I dedicate my two-year graduate experience and this work to all the people around me for supporting, guiding, helping and encouraging me in all my academic pursuits and dreams. To my loving parents, Yuping & Cuiying, my uncle, Joseph and my wonderful friends; this is for you all.

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Chapter 1 Introduction

1.1 Background

Approximately 48 million people are affected by food-related diseases every year in the United States. While fresh fruit and vegetables are an indispensable component of a healthy diet, contamination by foodborne pathogens due to improper handling can lead to serious diseases and even death. A recent report from the U.S. Centers of Disease Control shows that almost half (46%) of foodborne illness between 1998 and 2008 were attributable to fresh produce (Fig. 1.1 [1]). Preventing foodborne illness remains challenging. In recent years, significant attention has been given to assessing the risk of contamination during various stages of production in both pre-harvest growing and post-harvest handling steps. As recent reviews showed, reducing pathogen contamination during on-farm food production is a critical and efficient way to reduce foodborne illness incidence [2].

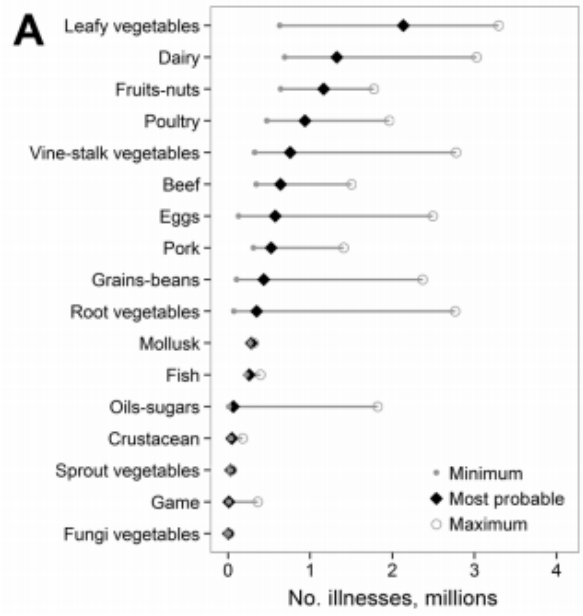

Figure 1.1 Estimates of the annual number of foodborne illnesses from all etiologies attributed to food commodities, United States, 1998–2008. Excerpted from [1].

The production of organic crops represents a rapidly growing agricultural sector (Fig. 1.2). Organic food sales in the United States have increased from approximately $11 billion in 2004 to an estimated $27 billion in 2012, accounting for more than 3.5% of the total food sales. The contamination frequency and enteric pathogen occurrence on organic produce have received less attention than conventional produce, in part because of the small-scale nature of its production. Contaminated produce from small farms would likely lead to localized illness, rather than large multistate outbreaks. The microbial safety of organic produce remains under-researched, especially for small organic farms, which often have limited resources to evaluate the microbiological safety of their produce. There are many differences between conventional and organic farming systems. In particular, instead of chemical fertilizer, organic farming relies heavily on animal manure, which is a well-known source of enteric pathogens [3]. Moreover, due to the limited selection and cost of post-harvest treatments approved for organic certification, most small-scale organic farms typically implement minimal post-harvest steps aimed at reducing microbial loads. These differences suggest that research with conventional crops may not be directly applicable to organic crops and that the food safety risks that do exist for organic produce are not fully understood.

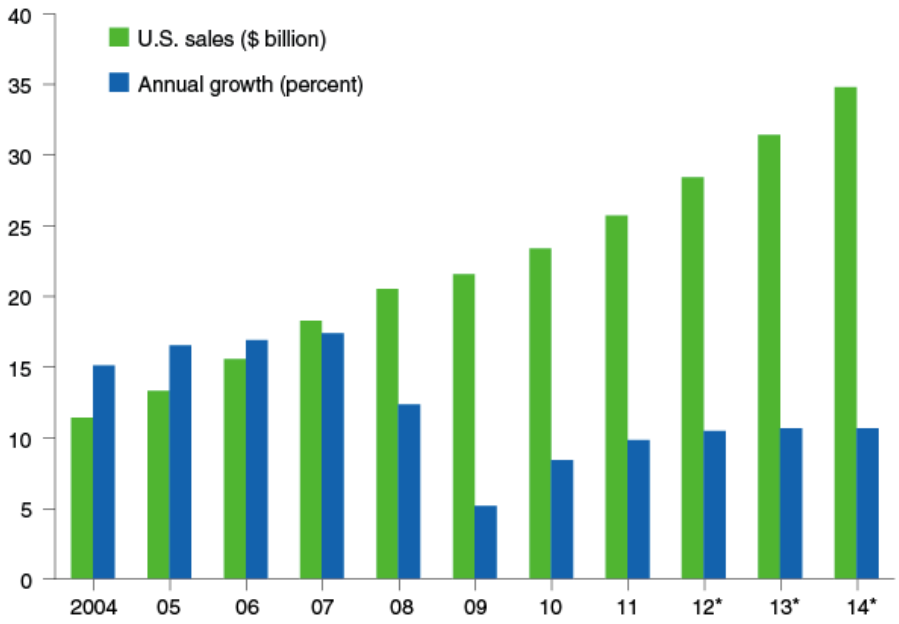

Figure 1.2 Organic food sales and annual growth in the United States during 2004-2013. Excerpted from USDA, Economic Research Service [4].

Despite the fact that organic produce makes up a very small percentage of food sales, organic produce recalls for food safety issues have occurred in recent years. For instance, in 2012, an organic spinach and spring mix product from Wegmans was recalled due to potential contamination with *E. coli* O157:H7, which is a pathogenic bacterium which can occur in produce and can cause severe illness and even death. In 2006, the same pathogen caused the first reported *E. coli* O157:H7 spinach outbreak, associated with contaminated Dole brand organic Baby Spinach, which caused 205 confirmed illnesses and three deaths [5].

Defined by USDA, organic food should be produced through approved methods that integrate cultural, biological, and mechanical practices. There are many limitations for crops, livestock and food in the organic standard. For instance, synthetic fertilizers, sewage sludge, irradiation, and genetic engineering may not be used. In the current study, some farms were certified, while others were not but followed organic farming systems.

The growing and handling practices adopted on small organic farms are different from conventional ones. In fact, they even differ among different organic farms. One example is the variety of ground cover that may be encountered for growing the same crop. For growing produce, many farms have adopted the use of plastic cover mulches and some farms have used corn-based plastic cover mulches to maintain soil warmth and humidity. Straw is another commonly used mulch. However, the impact of various mulches on food safety risk is not known.

For handling practices, a small survey conducted in 2013 (unpublished, Micallef, Martin and Pahl 2013) during the Maryland Organic Food & Farming Association (MOFFA) Annual Meeting, including 22 organic farmers in Maryland, showed that 43% of them washed produce in packing houses and 43% of them sometimes washed their produce. Most, 75%, used well water to wash the produce, but 6% of them used surface water and 19% municipal water for post-harvest rinsing. When asked about the use of sanitizers, 93% of them responded that they did not add sanitizer to the washing water. Most organic farms in Maryland have small-scale production and adopt a variety of practices. Handling methods also differ and the majority of them use minimal handling. There are significant gaps in our knowledge of the food safety risks associated with produce cultivated and handled on small-scale farms using organic farming systems.

1.2 Hypotheses of Research

The hypotheses of this research are:

i- The microbiological safety of post-harvest produce differs from pre-harvest produce;
ii- The adoption of different cropping practices, such as various mulches, affects the fate and survival of bacterial indicator species during cultivation;
iii- The bacterial communities of washed and unwashed lettuce will differ with sanitizer use and after 5 days of storage.

In other words, both pre-harvest growing methods, such as mulching, and post-harvest handling procedures, such as manual harvesting, post-harvest washing and storage may have an effect on the microbiological safety of produce from small organic farms. To completely understand the microbiological safety of produce, not only do we need to monitor the presence of pathogens and indicator microorganisms, but useful information can also be gained by analyzing the epiphytic bacterial communities.

1.3 Study Approach.

This study was conducted in three phases. In the first phase, microbiological safety of fresh produce samples from small Maryland organic farms was evaluated. In the second phase, the effect of mulching, a widely used growing method in produce farming, on survival of indicator bacteria on lettuce was evaluated. In the third phase, the impact of post-harvest washing method on the microbiological safety and epiphytic bacterial communities associated with lettuce was assessed.

1.4 Potential Impact of Study

This research study aims to evaluate the microbiological safety of organic crops produced on small farms in the mid-Atlantic, before and after handling. These data are indispensable to making informed decisions that could improve Good Agricultural Practices (GAPs) and Good Harvest Practices customized for small organic growers. This is especially pressing in view of the Food Safety Modernization Act (Public Law 111-353) that, in the wake of a food-borne outbreak, reserves the right to lift certain exemptions currently allowed for small farmers. Data from the assessment of cropping and post-harvest rinsing methods will provide science-based evidence to validate current metrics or suggest establishment of new recommendations.

Chapter 2 Literature Review

2.1 The Problem

Food safety has attracted wide attention in recent years, not only from government and policy makers, but also from the general public. Safety has become one of the most important attributes of food for consumers in the United States and Europe [6]. Concerns over food quality have significant influences on purchasing habits, food safety policies, as well as on the farming industry. For example, the fast expansion of the organic food market is partly due to effect of food safety perceptions. The U.S. sales of organic food increased from $3.6 to $26.7 billion between 1997 and 2010 [4]. According to surveys of consumer attitude and preferences [7–10], one important factor for preference towards organic food is health-related issues, and the notion of food safety has a particularly important influence for purchasing organic food.

Although organically grown food has generally been perceived as safer, there is actually limited scientific research supporting a difference between conventionally grown alternatives [10,11]. The pre- and post-harvest microbiological safety of organic crops, which impacts food safety and produce quality, has not been fully assessed. Data specific to organic produce and small scale production, aimed at improving agricultural and handling practices and minimizing human pathogen contamination of crops, are scarce. One study reported that no *Salmonella*, *Campylobacter*, *E. coli. E. coli O157*, *Listeria* were found in any of the organic vegetable samples [12], and a comparative study of organically and conventionally grown spring mix found no statistically difference [13].

Another study in Norway showed that organic leaf lettuce was generally of acceptable quality but contamination with *E. coli* and *L. monocytogenes* did occur occasionally [14]. Another study evaluated the microbiological safety of fresh-cut organic vegetable produced in Zambia [15].

In the Mid-Atlantic area, organic production remains small-scale. Crops grown organically on small farms are often sold at farmer's markets or Community Supported Agriculture (CSAs), are distributed over smaller geographical areas, and as such are unlikely to cause multistate outbreaks. As a result, contamination frequency and enteric pathogen occurrence of this agricultural sector has received less attention and microbial safety of organic produce from small farms remains under-researched. Nevertheless, the social and economic impact of organic farms on local communities they serve is considerable, reflecting the desire to support local farmers and buy locally grown food for health and environmental reasons.

In conclusion, there is a lack of data on the microbiological safety of organically grown produce on small- to medium-sized farms, which comprise a large proportion of farms in the Mid-Atlantic region of the U.S. Nevertheless, food safety risks for organically farmed fresh produce do exist. There is a need to fill in these data gaps since data from conventional crops are not directly applicable to organic crops

2.2 The Difference between Conventional and Organic Farming Systems.

2.2.1. The Pre-harvest Production Stage

There are several key differences between organic and conventional farming systems. "Organic" refers to the way farmers grow and process agricultural products. Organic farming systems ban the use of synthetic pesticides, fertilizers and herbicide, as well as growth regulators and livestock additives [16]. Instead, the organic farming system relies on animal manure-derived fertilizers and green manures, crop residues, naturally-derived pesticides and biological pest control methods to maintain soil productivity and promote sustainability. There is a general belief among consumers that environmentally friendly techniques will lead to the production of safe food [17]. However, scientific evidence in support of this perception is scare, despite a wealth of anecdotal reports.

One major criticism of organic food is whether the use of organic fertilization results in increased exposure to biological contaminants, which would put the consumer at a higher risk of foodborne illness. The use of animal manure, which is a well-known source of pathogens, has led to significant concerns [18,19]. Both conventional and organic agriculture use manure as a source of fertilizer. However, the manure application is generally much more intensive and widespread in organic farming systems since conventional farmers can use a variety of effective synthetic fertilizer. Although certified organic farms are restricted from using manure 120 days before harvest according to the USDA National Organic Program, pathogens may survive in the soil over a longer period, and could regenerate under favorable conditions, such as following field application [20]. The FDA Food Safety Modernization Act (FSMA) has proposed to extend this wait period to 9 months for crops that are likely to touch the soil, or 45 days if manure has been fully

composted. The most common treatment options to neutralize pathogens include anaerobic digestion, aerobic digestion, composting, and thermal depolymerization. Compost is considered fully composted and safe to use for fresh produce crops if the temperature reaches the desired level (55-75°C) and turned 5 times in a windrow system while monitoring that the C:N ratio is kept between 25:1 to 40:1. The compost must be kept at that level for a specific time in the whole batch of sludge [21] until either the density of *Salmonella* spp. in the compost is less than 3 MPN/4 g of compost dry weight, or the fecal coliform density are less than 1,000 MPN/g of compost dry weight [2]. One study showed that different mulches impacted this survival, with organic mulches having long-term effects on the soil bacterial communities [22], whereas without mulching, pathogens were not detected 12 weeks after manure inoculation.

The increased biodiversity and overall population of several species in organic farm systems may also serve as contamination sources [23,24]. In particular, organic farming uses different weed control methods to conventional farming. Instead of herbicide, many organic operations rely on manual labor and physical methods, such as a combination of mulching, mowing and cultivation for weed control [25]. While organic farming leads to enhanced biodiversity and soil fertility, contact with birds, rodents, reptiles and other animals may transmit fecal pathogens to produce [10,18]; although animal intrusion risks are also present in conventional farming systems.

The presence of fungi is another concern in food safety. Certain molds can produce a toxic and carcinogenic metabolite called mycotoxin. Since effective synthetic fungicides

are not permitted during the growing and processing of organic products, which may lead to higher risk of mycotoxin from molds. Moreover, due to lack of chemical fertilizer, the lower nitrogen in organic produce is likely to increase the sugar content [13], and make organic products more susceptible to fungal attacks. On the other hand, plough tillage has been shown to be effective at reducing fungal attack incidence [26], and tilling the soil between crop application is generally used as a weed control technique in organic agriculture since use of herbicide is prohibited. Therefore, it remains a controversial issue whether organic farming practice is more susceptible to fungal attack.

2.2.2. The Post-harvest Handling Stage

Significant differences exist between organic and conventional agriculture in the options for post-harvest handling and sanitization methods. Decontamination of food by using irradiation, chemical washes, a variety of antimicrobial agents or other synthetic disinfectants is prohibited in organic farming, while other practices, such as the use of chlorinated water and pasteurization are optional. The adoption of sanitization methods varies substantially among organic farmers [27,28]. It is likely not all organic farmers use post-harvest water sanitization methods.

When harvesting and packing in the field, the harvest bin and any container or tool should be kept clean and should be sanitized prior to use. After harvest, produce are sorted by hand or machines depending on the farm. Some produce types are washed on the farm. Chlorine (tap water), ozone, and peroxyacetic acid (PAA) are most common ways for washing organic produce. Chlorine (chlorinated in tap water) may be used,

within specified limits, 5ppm, in the forms of liquid sodium hypochlorite (bleach), granular calcium chloride within specified limits. PAA is a substance allowed to come in contact directly with produce according to NOS and therefore a good option for small and medium size organic farms. It has good efficacy in water dump tank and flume water sanitation applications in removing and controlling microbial biofilms in tanks and flumes although it is restricted to large bulk units [29] .

Storage is a very important stage after washing since temperature is the single most important tool for maintaining produce quality after harvest. The common ways include room cooling, forced-air cooling, hydrocooling, top or liquid icing and vacuum cooling. Clean packaging bags are required to prevent contamination before sale (OEFFA Organic Certification Fact Sheet). Farm managers should establish a protocol for cleaning and disinfecting harvest equipment, cleaning/processing facilities, and transportation. Organic producers, packers, and handlers are required to keep records of postharvest wash or rinse treatments, identified by brand name and source [29].

2.3 How Organic Produce Contamination Occurs

Although there have been many investigations into the prevalence and transmission of microbial pathogens in conventional farming systems, little information is available regarding the organic farming systems. The current view from the FDA is that there is currently no firm evidence to support the premises that organic produce is more or less microbiologically safer than conventional produce [30,31]. The majority of comparative studies find no significant differences in the bacterial counts of organically and

conventionally grown produce [32–36]. A few studies have detected several strains of *E. coli* and *Salmonella* more frequently in organic vegetables than conventionally produce [37]. It is clear that organic food is not immune to foodborne pathogen contamination, but the debate remains.

Fresh produce can be contaminated by food pathogens at various stages during the production cycle: during growth in the field; during harvesting; post-harvest handling; processing; shipping; marketing and final preparation by the consumer at home [18]. Generally the contamination can be roughly divided as occurring pre-harvest and post-harvest. Pre-harvest contamination primarily takes place at the site of production on the farm, whereas post-harvest contamination occurs after the produce has been harvested. This study considers both contamination during the pre-harvest stage and during post-harvest handling and processing. Several sources of contamination will be discussed in this section.

2.3.1 Sources of Pre-harvest Contamination and Good Agricultural Practices

There is a growing body of information on the potential contamination sources during cultivation of produce. Pre-harvest contamination can occur directly via contaminated manure and irrigation water or indirectly via wild animals, insects and human handling. Animal manure has been highlighted as one of the most important and direct sources of contamination [38,39]. Recent studies also identify animal activity and personal hygiene of field workers as two significant risk factors [39,40].

2.3.1.1 Fertilization

Animal manure is widely used as a crop fertilizer, especially in organic farming and in areas where livestock farming coexists with arable farming. A proportion of the animal manure could contain a variety of pathogens. The potential for pathogens to contaminate fresh produce crops, either following soil amendments or through accidental cross-contamination, leads to increased risk of infection in humans. USDA incorporated the Produce GAPs harmonized food safety standard into its GAP & GHP audit program in 2011. It is recommended that the time between application of manure to produce production areas and harvest should be maximized (USDA: Guide to Minimize Microbial Food Safety Hazards for Fresh Fruits and Vegetables). Although certified organic farms are restricted from using manures within 120 days of harvest, survival of microorganisms in manure, soil and water varies greatly, from days to as much as a year depending on the environmental conditions and microorganism. Another study showed that *E. coli* O157:H7 from contaminated manure can enter the lettuce plant through the root system and migrate throughout the edible portion of the plant [41]. Other studies found no difference in bacteriological quality at harvest after applications of different types of manure [42], and the transmission of *E. coli* O157:H7 from contaminated soil to lettuce did not occur [43].

2.3.1.2 Irrigation Water

Irrigation water has been identified as another important source of contamination. Water is critical both in the (pre-harvest) growth and (post-harvest) processing of crops.

Depending on the sources of water, there can be substantial variation in quality and safety. Irrigation water can be contaminated by pathogens from animal fecal deposits or contact with contaminated surface runoff [41]. Moreover, pathogens can survive in water for extended periods after contamination [44]. If contaminated water is used for crop irrigation, pesticide application or frost protection, it can introduce pre-harvest contamination to the produce. Indeed, a number of *E. coli* O157:H7 outbreaks have been linked with contaminated water.

A recent study in southern Brazil detected *E. coli* O157:H7 in irrigation and wash waters [45]. Two studies showed that *E. coli* O157:H7 contamination in soil persisted for more than 5 months after application of contaminated compost or irrigation water and the effects of irrigation water and manure were similar [46,47]. Another study showed both contaminated manure compost and irrigation water could contaminate soil and root vegetables with salmonellae for several months [48]. A recent study found that under growth chamber conditions, *E. coli* O157:H7 populations in irrigation water that complies with the Leafy Greens Marketing Agreement (LGMA) standards will not persist for more than 24 h when applied onto foliar surfaces of spinach plants [49].

2.3.1.3 Animal Activity

Intrusion of wild animal and cattle is another source of pathogen contamination. Animals could carry food-borne pathogens and contaminate crops directly via fecal deposition or indirectly through fecal contamination of soil or irrigation water (Jay-Russell, 2013). Wild animal intrusion may produce food safety risks at the pre-harvest level due to the

low infectious dose of many enteric pathogens (e.g., *Salmonella*). In particular, the strain associated with the highly publicized 2006 *E. coli* O157:H7 outbreak was isolated from domestic cattle and feral swine from adjacent rangeland. Fecal contamination of crops by animals is now considered a significant risk factors at the pre-harvest stage [40,50,51].

2.3.1.4 Field Workers

Another source of pre-harvest contamination that has been identified is the activities and personal hygiene of field workers. A recent study reported that generic *E. coli* was significantly reduced after field workers were trained to use portable toilets and hand-washing stations [39]. Worker training is an important part of GAPs and is required yearly. Better worker supervision can reduce the risk of contamination.

2.3.2 Post-harvest Contamination and Good Handling Practices

2.3.2.1 Factors Affecting Contamination after Harvest

Once crops are harvested, there is a series of physiological changes that occur to the produce. Mechanical disruptions during harvest will lead to changes in surface morphology, tissue composition and metabolic activities, especially when processed as "fresh-cut". These disruptions will give rise to a wide range of diverse ecological niches, which will be selective for specific species of microorganisms [52]. Operations such as cutting, shredding, dicing, and peeling will generate bruised and cut surfaces, which will then exude fluids that contains both nutrients and antimicrobials [53] and change the growth

of the microbiota and pathogens. The cut-surfaces also make the produce more susceptible to attachment and entry of pathogens. Previous studies have shown that plant lesions can promote rapid multiplication of *E. coli* O157:H7 on post-harvest lettuce [54]. Cross-contamination is another factor that should be considered in post-harvest contamination. Pathogens can be spread during washing and/or through the use of cutting devices. Another study found the cross-contamination of lettuce with *E. coli* O157:H7 via contaminated ground beef through cutting boards (*143*). Moreover, if containers are not cleaned and sanitized after every use, they may contaminate the next products that are placed in the container [56].

2.3.2.2 Post-harvest Rinsing

According to the 2011 USDA GHP program, appropriate washing methods should be used and the efficacy of washing treatments should be maintained. This should consider washing water temperatures to reduce contamination and prevent infiltration. The use of sufficient sanitizer in the water is critical to minimize potential cross-contamination. A variety of antimicrobial agents and synthetic disinfectants are prohibited for organic farming. A recent study evaluated the effectiveness of different antimicrobial plant extract-concentrate formulations on four types of organic leafy greens inoculated with *S. enterica* serovar Newport and found that the antimicrobial activity was both concentration and time dependent [57]. Nevertheless, for organic farming, the use of sanitizer is optional and therefore organic produce could be more susceptible to post-harvest cross-contamination than conventional produce.

2.4 The Pathogens

Over 250 types of pathogens and toxins can be transmitted by food, with 31 of them classified as major food-borne pathogens [58]. Three most common bacterial foodborne pathogens associated with fresh produce are reviewed in this section, namely *Salmonella*, Shiga-toxin producing *E. coli* (STEC), and *L. monocytogenes*.

2.4.1 *Salmonella*

S. enterica subspecies *enterica* is a gram-negative, rod-shaped bacteria commonly found in the gastrointestinal tract of both exothermic and endothermic animals, including humans. It is a member of the Enterobacteriaceae. *Salmonella* can be divided into serotypes based on antigens that the organism presents. Scientists have classified S. *enterica* into over 2,500 serotypes. *Salmonella* is the most commonly diagnosed and reported foodborne illness associated with fresh produce, causing 15.19 cases of illness per 100,000 people in the U.S annually [59]. Despite some recent progress in reducing *Salmonella* infections, infection rate is still well above the national goal for 2020, which is 4 cases per 100,000 people.

Salmonella has remarkable adaptability and high tolerance for environmental stress such as UV radiation [60,61]. *Salmonella* can be widely distributed in nature and survive well in a variety of food, such as poultry, eggs, dairy products and fresh produce [62]. It has also been found that *Salmonella* can persist in the environment for extended periods, and can cause infections after the ingestion of low doses, e.g., 10-100 cells [38]. Moreover, *Salmonella* can be carried in the intestines of domestic and wild mammals, birds, and

reptiles. It also presents in the feces of pets, such as cats, dogs, hamsters, and guinea pigs. These properties make it hard to control *Salmonella* contamination.

2.4.2 Shiga-toxin Producing *E. coli* (STEC)

Unlike most strains of *E. coli* that are benign inhabitants of the gastrointestinal tract of endothermic animals, *E. coli* O157:H7 is a Shiga-toxin producing *E. coli* (STEC) that presents in the feces of livestock and wild animals. It was first identified as a human pathogen after two outbreaks associated with uncooked hamburger patties [63]. These organisms can produce Shiga-toxins, encoded by the genes stx_1 and stx_2. Infection of *E. coli* O157:H7 through consumption of contaminated food may lead to severe, acute hemorrhagic diarrhea, and to kidney failure. A 2006 outbreak linked to Dole bagged baby spinach caused more than 200 people to become ill and at least 30 to develop hemolytic uremic syndrome (HUS), a serious and potentially fatal kidney pathology associated with Shiga-toxin producing *E. coli* infections [5].

Surveys in the United States and Canada indicate wide spread distribution of *E. coli* O157:H7 in cattle operations [64]. *E. coli* O157:H7 may be present in animal manures and slurries, particularly cattle derived material [65], and can contaminate fresh produce during manure application. Wildlife such as deer, may also be carriers for *E. coli* O157:H7 [66].

2.4.3 *L. monocytogenes*

L. monocytogenes is one of the most virulent foodborne pathogens with 20-30% of infections in high-risk individuals resulting in death [67]. *L. monocytogenes* can be found in soil, which can lead to vegetable contamination. Animals can also be carriers of *L. monocytogenes*. *L monocytogenes* can cause a rare but severe disease, listeriosis. A number of surveys report the presence of *L. monocytogenes* on fresh produce such as cucumber, peppers, potato, radish, leafy vegetables, beansprout, broccoli, tomato and cabbage [53], with variation of prevalence on different types of produce. It has also been shown that *L. monocytogenes* can grow on lettuce when exposed to processing conditions [68] and during storage at refrigeration temperatures [69]. Infections are largely associated with the ingestion of high dose, e.g., 10^6 cells [70] by fetuses, neonates, and individuals with compromised immune system. As a result, outbreaks linked to fresh produce are infrequent. In the United States, *L. monocytogenes* is responsible for an estimated of 1,600 illness and 260 deaths every year. Recently, a cantaloupe linked listeriosis outbreak has caused 147 confirmed cases and 33 deaths across U.S states, which is the worst foodborne illness outbreak in the United States as measured by the number of deaths [71].

2.5 The Epiphytic Bacteria Communities and Pathogens

The phyllosphere represents a biome that is normally colonized by a diverse set of bacteria, fungi and other microorganisms [72]. Most of the microbial species that can be isolated from the above-ground parts of healthy plants are on the plants' surfaces rather than the within-plant tissues. Bacteria are being found on leaves in number up to 10^8 cells/g of leaves [73–75]. Only a very small fraction of the epiphytic bacteria is pathogenic for humans. The relationship between composition of bacterial communities and survival

of pathogenic bacteria on leaves represents a complex issue that is poorly understood. Recent studies revealed that each type of produce has its distinct epiphytic bacterial community profile [76,77]. Different post-harvest handling processes (e.g. washing, storage) can significantly change the composition of epiphytic bacterial communities. It remains to be explored whether the attachment, survival and growth of enteric pathogen would be altered.

2.5.1 Antagonistic Interaction between Phytobacteria and Human Enteric Pathogens

Biological control (biocontrol) is a pathogen management strategy that uses micro-or macro- organisms to suppress or eliminate pathogens without use of chemicals [78]. Some species of biocontrol microbes can inhibit other microorganisms directly by producing biocides, antimicrobials, and exoenzymes or competing for nutrients, or indirectly by inducing plant defenses to reduce fitness of other organisms [78,79]. Several studies have demonstrated that phytobacteria can significantly reduce the growth of human enteric pathogens. A strain of *Pseudomonas syringae* was shown to reduce the growth of *E. coli* O157:H7 from wounded apples by 10-1000 fold [80]. Using *in vitro* agar spot bioassay method, [81] showed that *Pseudomonas fluorescens* 2-79, a plant-associated pseudomonad, produced clear zone of inhibition against *S. enterica* and reduced the growth of *Salmonella* on alfalfa sprouts by approximately 100,000 times [82]. *Enterobacter asburiae*, another type of epiphytic bacteria, was also found to be effective in reducing *E. coli* O157:H7 and *Salmonella* on lettuce and *Arabidopsis* [83], likely by competition for carbon and nitrogen sources [84].

2.5.2 Human Enteric Pathogens Benefit from Interactions with Phytopathogens

Although some species of phytobacteria can suppress growth of human enteric pathogens, others can promote the attachment and growth of human pathogens [79,84,85]. There are a number of studies showing that human enteric pathogens can benefit from interactions with the presence of plant pathogens [54,86–90]. In particular, post-harvest decay of produce can lead to an increased risk of contamination by enteric pathogens [79]. A survey on produce sample from New Jersey revealed that at least 60% of the samples affected by bacterial soft rots were positive for *Salmonella* [87]. A follow-up study revealed that promotion of *Salmonella* growth is specific to some species of decay-inducing fungi: 20–30% of produce damaged by *Alternaria* or *Botrytis* tested positive for *Samonella*, but only 1–2% of produce damaged by *Geotrichum*, *Sclerotinia*, *Stepmylium*, *Thielaviopsis* tested positive for *Salmonella* [87]. It is also noted that *Salmonella* is detected in 2-8% of the mechanically damaged samples. The damage to the plant by fungi and mechanical forces may provide sites for the survival and growth of pathogens.

How enteric pathogens benefit from associations with phytopathogens remains unclear. One hypothesis is that these human enteric pathogens can benefit from pectinolytic activities of plant pathogens [91]. Published genomes of *Salmonella* Typhimurium and *E. coli* O157:H7 contain homologues of the genes involved in the uptake of compounds that result from pectin degradation [92]. It is possible that these human enteric pathogens can take up uronic acids and other compounds that result from the pectinolytic activities of plant pathogens ([79]. Moreover, the increase of pH from 4 to 5-6 when plant tissue is

degraded by macerating phytopathogens may also be beneficial for the growth of enteric pathogens [90,91,93].

2.6 Knowledge Gap

Fresh produce is susceptible to contamination by foodborne pathogens at many stages from production at the farm to preparation in the home. Extensive research has been conducted to investigate sources of contamination in both pre- and post-harvest settings. An increasing amount of knowledge is known about sources of pathogen in the environment, vehicles and routes of transmission, detection of pathogens on pre-harvest produce, microbial ecology of produce, etc. It has been increasingly recognized that an integrated strategy to reduce foodborne illness associated with fresh produce requires steps to mitigate pathogen contamination during on-farm food production [94]. Despite the progress, how and where does pathogen contamination occur in the farm-to-fork chain is often unknown. There is a need to better understand the dissemination, survival, and growth of foodborne pathogens both in the field and during post-harvest handling stages. Moreover, although there is a wealth of data from conventional farming systems, microbiological safety and food safety of organic crops has not been fully assessed. Specifically, little data exists on the difference in food safety between in field and harvested produce. The harvesting process and immediate post-harvest processes need to be assessed for food safety risk. Data and educational resources specific to organic production aimed at improving agricultural and handling practices and minimizing human pathogen contamination of crops are scarce. There is a need to fill in this data gap

and evaluate factors that may have an impact on microbiological safety on organically grown fresh produce in both pre- and post-harvest stages.

2.6.1. Impact of Cropping Methods on Pathogen Fate and Survival

Cropping methods are important factors not only for the characteristics and nutrients of the produce, but may also have an impact on the food safety risk assessment. A variety of cropping methods are used to grow lettuce in the Mid-Atlantic region of the U.S., but the influence on enteric foodborne pathogens remains largely unexplored on small-scale organic farms, which typically have few resources for systematic product testing. There is increasing amount of information on the potential of in-field contamination from direct sources such as manure [43] and irrigation water and indirect sources such as animal activity. However, to completely assess the risk of contamination, we not only need to identify the sources of contamination, but also need to evaluate the impact of methods of cultivation.

Mulching is a cropping method widely used for lettuce cultivation. Mulches bring many benefits to the production of fresh produce. Studies have shown that mulching has significant effects on lettuce's head, leaves and stem growth, as well as the total yield increased with mulch compare with bare ground [95]. Plastic mulches can directly affect plant micro-climate by decreasing soil water loss and modifying surface radiation budget [96], thus providing high productivity and values for the amount of nutrients accumulation in the lettuce [97]. However, little is known about the effect of mulching on the survival of foodborne pathogens. Previous studies have shown that organic mulches could have long-

term effect on the soil bacterial communities [22], while without mulching, pathogens were not detected 12 weeks after inoculation [43]. Little is known about how mulch types influence pathogen persistence. For example, one study showed that straw mulch reduced levels of center rot on sweet onion, while black plastic mulches had the opposite effects, speeding up the onset of the epidemic [98].

2.6.2 Impact of Harvesting and Post-harvest Treatments on Microbiological Safety

The phyllosphere represents a complex biome that is normally colonized by a diverse set of bacteria, fungi and other microorganisms [72]. The majority of the epiphytic bacteria are harmless for human. As a result, most studies on the microbiological safety of fresh produce focus on the detection of major types of foodborne pathogens, such as *Salmonella*, *E. coli* O157:H7, and *L. monocytogenes*. However, a number of studies showed that the presence of phytobacteria and phytopathogens could have significant effects (either antagonistic or facilitatory) on the attachment, survival and growth of human enteric pathogens [78,79,84,85]. A better understanding of the epiphytic bacteria communities and how they are affected by various growing and post-harvest handling procedures is necessary to thoroughly evaluate the risk of foodborne pathogens.

Chapter 3 Project Objectives

The ultimate goal of this study was to assess what pre- and post-harvest factors impact the microbiological safety of organically cultivated produce on small farms in Maryland. Data from small scale production are scarce and data for produce from organic farming systems of this size are lacking. In this study, the effects of select pre-harvest practices (e.g. irrigation water source, ground cover) and post-harvest practices (e.g. worker handling, washing methods) on epiphytic microorganisms and survival of indicator bacteria on produce and leafy greens were investigated. There is a general lack of understanding of long-term impact of the various cultivation and handling practices adopted by these non-conventional growers. For example, in a 2008 study by Allende et al., [99], it was shown that while washing reduces microbial loads initially, its effectiveness is no longer significant when tested after 5 days of storage.

To address these knowledge gaps, the current study had three objectives, all related to assessing the pre- and post-harvest microbiological safety of organic produce, and the impact of growing and handling methods on epiphytic bacteria on Maryland farms.

1. The first objective was to determine the impact of manual harvesting and minimal post-harvest processing on the microbiological safety of fresh produce cultivated on small organic farms in Maryland. The prevalence of pathogens on organic produce pre- and post-harvest: *S. enterica*, Shiga toxin-producing *E. coli* and *L. monocytogenes*, was assessed. This included an assessment of potential role of irrigation water as a source of

these foodborne pathogens. Microbiological safety was also assessed by quantifying the concentrations of potential indicator microorganisms, including generic *E. coli*, total coliforms, aerobic bacteria (APC) and yeast and molds, that are also associated with conditions with increased risk pathogenic bacteria (see above).

2. The second objective was to evaluate the effect of different mulches on *E. coli*, *Enterococcus* spp. and fecal coliforms persistence on lettuce grown under organic practices by contrasting different mulches with cultivation on bare ground.

3. The third objective was to compare the prevalence of selected indicator microorganisms (total coliform, *E. coli,* APC, yeast and mold) and bacterial communities associated with post-harvest, washed (with three different treatments) and unwashed lettuce immediately following harvest and handling and after 5 days in storage.

Chapter 4 Microbiological Assessment of Organic Pre- and Post-harvest Fresh Produce and Irrigation Water from Maryland Farms for *Salmonella, L. monocytogenes* and Shiga Toxin-Producing *Escherichia coli* and Epiphytic Indicators

4.1 Introduction

A large proportion of organic production in the Mid-Atlantic region of the U.S. is from small to medium sized farms that sell to local retail markets, farmers' markets, and CSAs. There are 33 certified organic farms in Maryland and the total land cultivated is 303 acres. The total sale was $3,073,242 in 2011 (2011 Certified Organic Production Survey USDA, National Agricultural Statistics Service). There are 24 farms growing tomatoes with 4,899 cwt sale quantity. There are 4 farms growing spinach: with 253 cwt sale quantity. There are 18 farms growing lettuce with 659 cwt sale quantity. As for bell peppers, there are 16 farms growing lettuce with 1,715 cwt sale quantity.

Organic produce cultivated on small scale farms is unlikely to cause big multistate foodborne outbreaks. Many of these smaller farms harvest produce one to a few days before sale, and processing of produce post-harvest is typically minimal. Post-harvest handling varies widely from farm to farm and by produce type - ranging from direct field packing to post-harvest rinsing on a packing line, with or without the use of sanitizers in rinse water. However, there are insufficient data to address the question of microbial quality and safety of organically grown produce. Few studies point to microbiological differences between pre- and post-harvest, and at retail. Possible factors, such as irrigation water, that could affect the microbial quantity are still unclear. Only one study

on cantaloupe concluded that microbial loads originating from river water may survive on the rind or re-infest cantaloupes after the post-harvest processes (disinfection and rinsing) at the packinghouses [100].

This study aims to better assess the impact that harvesting and post-harvest handling practices have on microbial safety and quality of organically-grown produce from small organic farms. First, the prevalence of pathogens on organic produce pre- and post-harvest were determined and compared: *S. enterica*, Shiga toxin-producing *E. coli* (STEC) and *L. monocytogenes*. Secondly, irrigation water as a potential source of these foodborne pathogens was examined. Finally, the prevalence and levels of potential indicator microorganisms that could be used to assess pre- and post-harvest microbiological safety were compared.

4.2 Material and methods

Farm recruitment and sample collection

The study was conducted over a period of 2 years (2012 to 2013). Seven organic farms across the state of Maryland were recruited by invitation and willingness to participate. At each farm visit, a short survey was administered to obtain information on general farm-related management. The farm owner or manager was asked the questions in a face-to-face interview during each farm visit with follow-up emails for any clarification when management record referral was required to answer questions. Questions covered pre- and post-harvest practices including methods and water type used for irrigation, mulch type used for each produce, post-harvest handling practices, water type used to wash

produce and type of sanitizers used, if any. This information was used to identify potential associations between microbiological results and specific management practices (Appendix I).

A total of 179 produce samples (including vine stalk fruits and vegetables, bulbous vegetables, leafy greens and fresh herbs) and 27 irrigation water samples (well water and surface water) were collected during the summer and fall. Each farm was visited two to four times over the 2-year study period. At each farm visit, one to six types of produce from the field (pre-harvest produce samples) and the packaging area (post-harvest produce samples) were collected. Sterile gloves and scissors were used and changed between sample types. Leafy green samples consisted of composite samples of leaves from four plants. Vine fruit consisted of tomato, pepper, eggplant, zucchini, beans, okra, squash, grapes, and cucumbers. Samples were placed in sterile Whirl-Pak bags (Nasco, Fort Atkinson, WI). Irrigation water samples were collected from the lines or ponds using sterile 500 ml bottle. Almost 500 ml water sample were collected in the bottle after the line opened for 1min. From the pond, 500ml water from top level was collected, being careful not to disturb the sediment. All samples were transported in coolers with ice packs to the Department of Plant Science and Landscape Architecture laboratory at the University of Maryland and kept at 4°C until analysis. All water samples were processed within 24 h and produce samples within 48 h of collection.

Quantification of indicator microorganisms

Water samples: Shortly before testing, water samples were resuspended by shaking to evenly disperse microorganisms throughout the water in the bottle. Water was serially

diluted (10^0,10^{-1},10^{-2}) in 0.1% Peptone Water (PW) (Becton, Dickinson and Company (BD), Sparks, MD), and 200 ml of each dilution was filtered through sterile 0.45 µm pore size membrane filters. Filters were aseptically removed from the funnel, placed on MI agar plates (BD) and incubated for 20-24 h at 35 ± 0.5°C for enumeration of *E coli* and total coliforms. After incubation, the number of blue or indigo colonies under normal/ambient light was counted to obtain *E. coli* counts. The number of fluorescent colonies under long-wave ultraviolet light (365 nm) and the *E. coli* count were summed together to get the total coliform count (TC).

Produce samples: Each sample was weighed and an equal volume of 0.1% PW was added to each bag, except for leafy green samples, where the weight/volume ratio was 1:10. Leafy green bags were stomached in a stomacher (Seward, Stomacher 400 circulator, U.K.) for 2 minutes at 250 pulses per min at room temperature. Vine stalk and bulbous vegetables were gently hand-rubbed for 2 min. Serial 10-fold dilutions were prepared in 0.1% peptone water (PW) (BD). The levels of total coliforms and *E. coli* in all samples were enumerated by duplicate 1-ml samples of appropriate dilutions spread plated onto 3M™ Petrifilm™ *E. coli*/coliform count plates (3M, St. Paul, MN.) and incubated at 37±0.5°C, as per manufacturer's instructions. Red colonies with gas bubbles observed at 24 h were counted as coliforms and blue colonies with gas bubbles observed at 48 h were counted as *E. coli* colonies, according to standard TC/*E. coli* Petrifilm enumeration methods. Appropriate dilutions were also plated directly onto 3M™ Petrifilm™ Aerobic Count Plates (APC) (3M), incubated at 37±0.5°C, and observed for red colonies after 48 h for enumeration of aerobic mesophilic bacteria. Yeast and Mold (Y&M) Count plates (3M) were plated and incubated at room temperature for 3-5 days. Small green colonies

were counted as yeast colonies and big green or brown ones were recorded as mold.

Pathogen isolation: All samples were enriched for isolation of *S. enterica*, Shiga toxin-producing *E. coli* (STEC) and *L. monocytogenes*. Three sub-samples of 200 ml of each water sample were filtered through membrane filters as described previously. Filters were placed in separate tubes containing 25 ml buffered peptone water (BPW) (BD), Brila Broth (EMD Chemicals Inc., Darmstadt, Germany) and Buffered Listeria Enrichment Broth (BLEB) (BD). For leafy green sample analysis, leaf samples were weighed and buffered peptone water, BB and BLEB added separately at the rate of 1:10. For all other produce samples a 1:1wt/vol ratio was used.

Samples in BPW were incubated at 37 ± 0.5°C for 24 h for *Salmonella* pre-enrichment. One ml aliquots of BPW suspensions were transferred to tubes containing 15 ml of Tetrathionate (TT) Broth base, Hajna (BD) with 1.2 ml iodine solution, vortexed and incubated at 37 ± 0.5°C for 24 h for selective enrichment. One 10 µL loopful from each enrichment tube was streaked onto XLT4 Agar plates (BD) and incubated for 24 h for presumptive identification of *Salmonella* colonies, distinguished as black colonies on the plates. In the absence of black colonies, TT Broth cultures were incubated for 5 days at room temperature and streaked again onto XLT4 Agar plates. Samples in BB were incubated at 44 ± 0.5°C for 6 h for STEC enrichment. Loopfuls of BB were streaked to CHROMagar™ STEC plates (CHROMagar, Paris, France) and incubated for 24 h for growth of presumptive STEC colonies, appearing as purple or mauve colonies on the plates. *L. monocytogenes* isolation, samples were enriched in BLEB by incubating at 30 ± 0.5°C for 4 h. Acriflavine HCl, nalidixic acid and cycloheximide were added to BLEB bag according to the BLEB base media directions and incubated at 30 ± 0.5°C for 20 h.

One 10 µL loopful from each enrichment bag was streaked onto CHROMagar™ Listeria plates(CHROMagar, Paris, France) and incubated for 24h for growth of presumptive *L. monocytogenes* colonies, distinguished as green colonies on the plates.

Isolate identification: The presumptive isolates of three food pathogens from above produce samples were archived in Brucella Broth with 20% Glycerol in tubes at -80°C. DNA was extracted from pure cultures on Tryptic Soy Broth (TSB) in logarithmic growth using UltraClean™ Microbial DNA Isolation Kit (MO BIO Laboratories, Inc., Carlsbad, CA), as recommended by the supplier. Genomic DNA isolated from samples (four to eight isolates per sample) was subjected to PCR amplification with primers specific to the coding region of the Bacterial 16S rRNA gene (*31, 84*) as an internal amplification control using primers and pathogen-specific primers intimin adherence protein (*eae*) gene, Shiga toxin-1 (stx_1) primers and Shiga toxin-2 (stx_2) primers [103], β-D-glucuronidase (*Uid*A) gene[104], hemolysin A (*hlyA)* [105], primer for STEC presumptive isolates identification and invasion-associated protein (*iap)* [106] primer for *L. monocytogenes* presumptive isolate identification (table 4.1) Primers used in PCR reactions have been previously described.

Table 4-1 Gene information for pathogen identification.

Taxon	Gene name	Sequence	Reference
Bacteria	16S rRNA	F: 5'- AGAGTTTGATCCTGGCTCAG -3'; R: 5'- CTGCTGCCTC CCGTAGG -3'	[12,107]
STEC	*eae*	F: 5'CATTGATCAGGATTTTTCTGGTGATA-3'; R: 5'- CTCATGCGGAAATAGCCGTTM -3'	[108]

	stx$_1$	F: 5'- GTGGCATTAATACTGAATTGTCATCA -3'; R: 5'- GCGTAATCCCACGGAC TCTTC -3'	[103]
	stx$_2$	F: 5'- GGCACTGTCTGAAACTG CTCC -3'; R: 5'- TCGCCAGTTATCTGACATTCTG -3')	[103]
E. coli	*uid*A	F: 5'- CAGTCTGGATCGCGAAAACTG -3'; R: 5'- ACCAGACGTTGCCCACATAATT -3'	[104]
Listeria	*hlyA*	F: 5'- GCAGTTGCAAGCGCTTGGAGTGAA -3'; R: 5'- GCAACGTATCCTCCAGA GTGATCG -3'	[105]
	iap	F: 5'- AATCTGTTAGCGCAACTTGGTTAA -3'; R: 5'- CACCTTTGATGGACGTAATAATACTGTT -3'	[106]

PCR was carried out in a total volume of 30 µl reaction mixture containing 1 µl of 10× Standard Taq Reaction Buffer (BioLabs Inc., New England), 0.8 U Taq DNA polymerase (BioLabs), 2 mM $MgCl_2$, 0.2 µm of each dNTP (BioLabs), 0.2 µm of each reverse and forward primer and 50–100 ng pure DNA. The remaining volume was adjusted by adding an appropriate amount of sterile ultrapure water. DNA was amplified through 30 cycles of denaturation, annealing and polymerization in a C1000Touch™ Thermal Cycler (BIO RAD, Singapore). Initially, DNA denaturation was carried out at 95°C for 2 min followed by 30 cycles of denaturation at 95°C for 15 s, annealing at 58°C for 30 s and extension at 72°C for 30 s. Amplified DNA fragments were analyzed on 1% (w/v) agarose gel (Lonza, Rockland, ME) in Tris–borate–EDTA buffer (BIO-RAD). The amplified DNA fragments were visualized using a Molecular Imager Gel Doc™ XR+

with Image Lab™ Software (BIO-RAD).The size of DNA fragments was established from molecular weight markers included in each gel. All samples were analyzed twice.

Statistical analyses: The software Matlab (R2013a, Mathworks) and JMP® Pro 10.0.2 were used for statistical analyses. A logarithm transformation were performed on the raw data using the formula $\log_{10}(1+c)$, where c is the measured bacteria count in the unit of cfu/g. Student's t-test, ANOVA, Tukey's test and Chi Square test were performed to determine whether the levels of contamination of different groups differed significantly ($p \leq 0.05$).

4.3 Results

A total of 208 samples were collected during the summer and fall of 2012 and 2013 from 7 farms of which five were certified and two were non-certified but adopting organic practices. A total of 29 water samples and 179 produce samples were analyzed. No *Salmonella* and *L. monocytogenes* were detected in any of the produce or water samples. Presumptive positive samples on Chromagar STEC were detected from 4 produce samples and 4 water samples (Table1). When analyzed for target genes, these isolates were found to be stx_1 and stx_2 negative, but *eae* positive. The positive samples consisted of one tomato, one chard, one pepper and three surface water samples from Farm 4, one cucumber sample from Farm 1, and one surface water sample from Farm 3. All the produce were pre-harvest samples.

Water Quality

Of the 29 irrigation water samples, 18 were groundwater samples collected from well taps and 11 were surface water samples collected from ponds (n=9) and a creek (n=2). The number of positive surface water samples was higher than groundwater samples for all bacterial indicators *E. coli and* TC. The prevalence for *E. coli and* TC in groundwater and surface water samples was, respectively, 4/18 (22.2%) and 6/11 (54.5%) for *E. coli*, 12/18 (66.7%) and 11/11 (100%) ($p<0.05$) for TC. The mean counts of the three bacterial indicators were all significantly higher in surface water compared to groundwater (Figure 4.1). Concentrations in groundwater and surface water respectively were 0.017 log cfu/100ml and 0.35 log cfu/100ml ($p=0.0097$) for *E. coli,* 0.36 log cfu/100ml and 2.19 log cfu/100ml ($p=2.6e-7$) for TC.

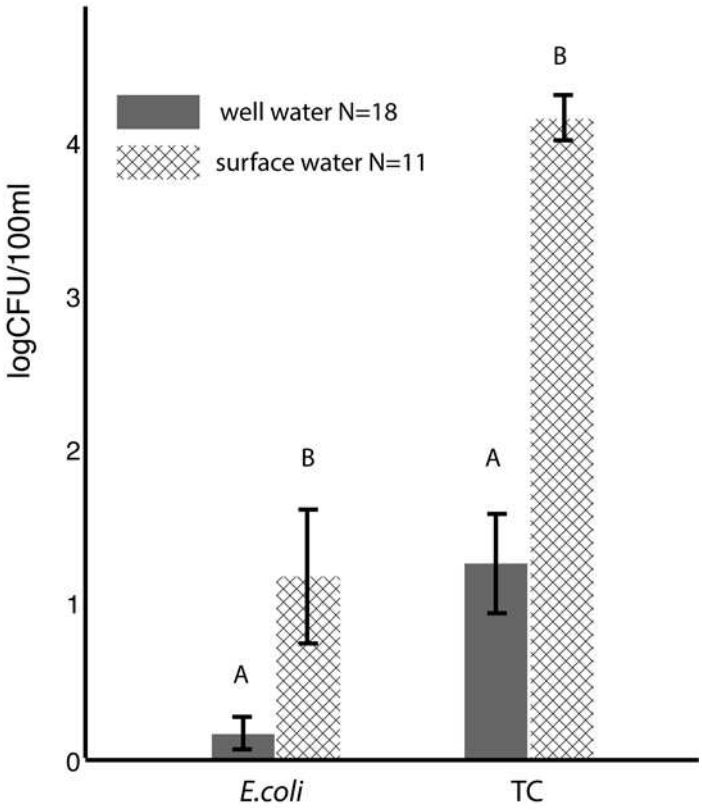

Figure 4.1 Geometric mean levels of *E. coli*, and total coliforms (TC) microorganisms in irrigation water samples. (Data of water types having different letters (A and B) were significantly different (P <0.05).

Produce Types

The 179 produce samples collected consisted of 17 commodities, and divided into 83 pre-harvest and 96 post-harvest samples. Among the samples, there were 42 tomatoes (23 pre-harvest, 19 unwashed post-harvest), 33 peppers (19 pre-harvest, 12 unwashed post-harvest, 2 washed post-harvest), 64 leafy greens, which consist of lettuce, chard, basil, spinach, kale, mustard (33 pre-harvest, 1 unwashed post-harvest, 30 washed post-harvest), and 40 other commodities (8 pre-harvest, 20 unwashed post-harvest, 10 washed post-harvest. These consisted of eggplant, cucumber, mizuna, okra, onion, squash, grape, turnip, zucchini, carrot and green bean samples). Among the produce samples, the

numbers and percentages of positive indicators in different produce types are listed in Table 4.2.

In pre-harvest produce samples, generic *E.coli* was detected on only two tomatoes and one leafy green samples. In terms of TC, tomato had the highest positive percentage (70%), and the lower ones were: leafy greens (63.6%), others (62.5%) and pepper (42.1%)(p=0.1658). In terms of APC, all samples were positive and almost all samples were positive for Y and M (Figure 4.2).

The levels of the five indicator microorganisms on pre-harvest leafy greens were higher than tomatoes, peppers and other produce types, with average counts in log(cfu/g) detected being respectively *E. coli*: 0.026, 0, 0.082 and 0 (non-detected); TC: 1.61, 0.832, 2.61 and 1.58; APC: 4.95, 4.95, 6.89 and 5.83; Y: 3.63, 3.41, 6.02 and 3.29; and M: 2.67, 2.37, 4.22 and 2.10. TC ($p=0.013$), APC ($p<10^{-5}$), Y ($p<10^{-5}$) and M ($p<10^{-5}$) were significantly different (Figure 4.2).

Comparison of Pre- and Post-harvest Produce

To compare the pre-harvest and post-harvest produce, produce samples were separated into four produce types and categorized into pre-harvest, unwashed post-harvest and washed post-harvest groups. A larger percentage of pre-harvest tomatoes were *E. coli* positive compared to unwashed post-harvest tomatoes. No *E. coli* was detected in peppers. Only one pre-harvest leafy green sample and two unwashed post-harvest other produce samples were positive. No *E. coli* was detected in washed post-harvest produce. As for

TC indicator, the positive percentage of pre-harvest tomatoes were higher than post-harvest ones (unwashed) and positive percentage of pre-harvest leafy greens were significantly less than washed post-harvest ones ($p<0.05$). For peppers and others, positive percentage of pre-harvest leafy greens was less than unwashed post-harvest ones and washed post-harvest ones were the highest among these three groups. As for the APC indicator, almost all the samples were positive, except four unwashed post-harvest produce samples. As for the yeast indicator, the positive percentage from low to high was: unwashed post-harvest, washed post harvest, and pre-harvest but there were only small differences ($p>0.05$). The positive percentage for molds for pre-harvest tomatoes was significantly higher than unwashed post-harvest tomatoes ($p<0.05$). Leafy greens showed an opposite pattern: pre-harvest less than post-harvest. The peppers and others vegetables had a higher frequency among the pre-harvest samples than post harvest, and washed was larger than unwashed post-harvest samples. All in all, for tomatoes, the positive percentage of pre-harvest is higher than unwashed post-harvest. For peppers, the percentage from low to high: pre-harvest, unwashed and washed post-harvest, and for leafy, washed post-harvest is higher than pre-harvest.

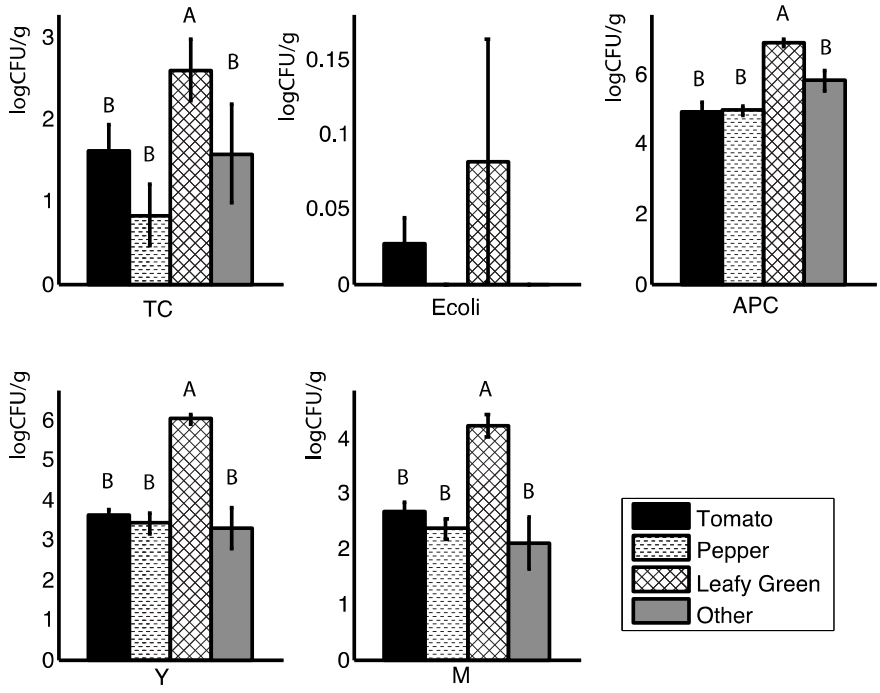

Figure 4.2 The quantity of bacterial indicator microorganisms in different pre-harvest produce type. Data of water types having different letters (A and B) were significantly different ($p < 0.05$)

All five of the indicator microorganisms were higher in pre-harvest tomatoes than unwashed post-harvest tomatoes. As for leafy, washed post-harvest is higher than pre-harvest, in which it is specifically significant in terms of Mold indicator ($p=0.03$). As for peppers and other produce types, washed post-harvest produce was higher than unwashed post-harvest (Figure 4.3).

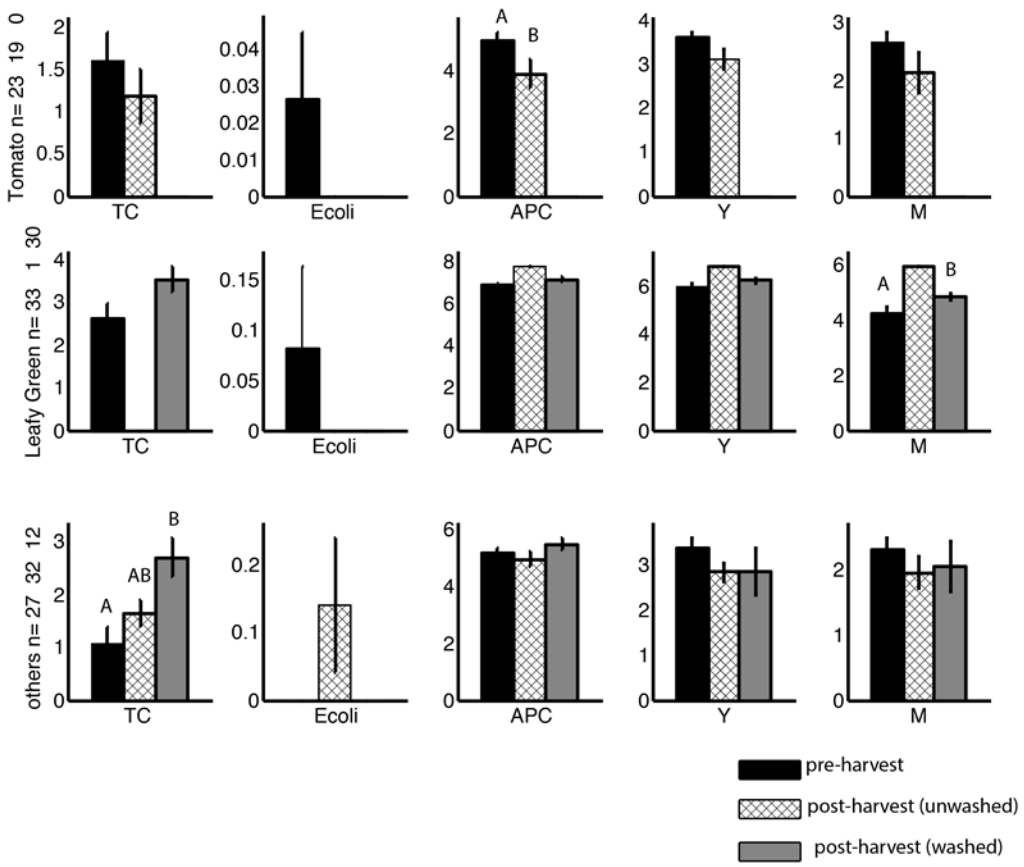

Figure 4.3 The quantity of bacterial indicator microorganisms in different produce types. (Data of produce types having different letters (A and B) were significantly different $p < 0.05$)

Correlation between irrigation water and produce

No correlation between the levels of *E. coli* and TC in irrigation water and produce was observed (Figure 4.4). The R-value and p-value in indicators of *E. coli* and TC are -0.1028 (p=0.7506) and 0.0122 (p=0.9699). There was a similar lack of correlation with tomatoes, with observed R-values and p-values for TC and APC of 0.0005 (p=0.9991) and 0.4059 (p=0.3184), respectively. The R-value and p-value for peppers for TC and

APC are 0.9598 (p=0.1812) and 0.0949 (p=0.9395). There are no values in terms of *E. coli* of tomatoes and peppers because not *E. coli* prevalence was too low.

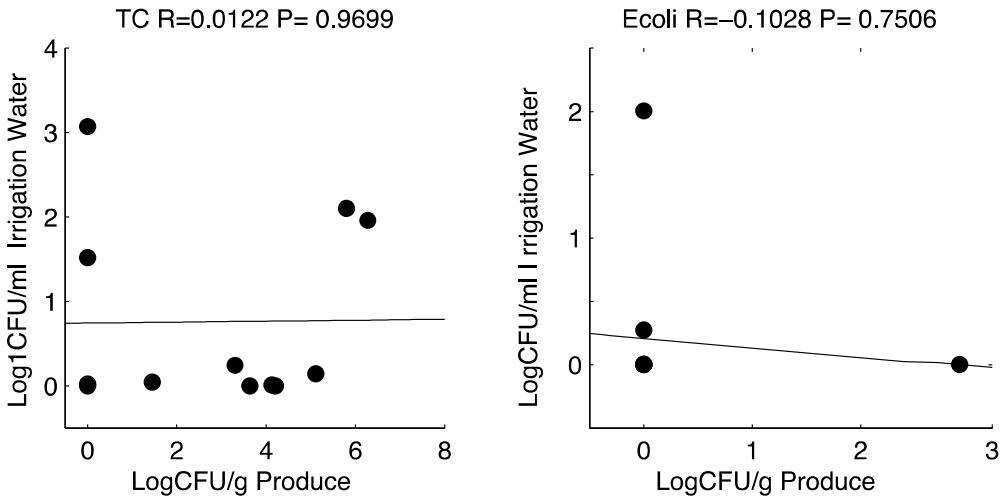

Figure 4.4 Correlation between bacteria levels in irrigation water and pre-harvest leafy greens (The point (0,0) in the right figure has 9 points in the same position.)

No significant difference among the farms in post-harvest produce, but the bacteria quantity of pre-harvest produce in farm1 and farm 3 are significantly higher than other farms in terms of TC and APC counts (Figure 4.5). The information for all seven farms is shown in Table 3. There are no significant differences between the same produce type using different mulches by different farms.

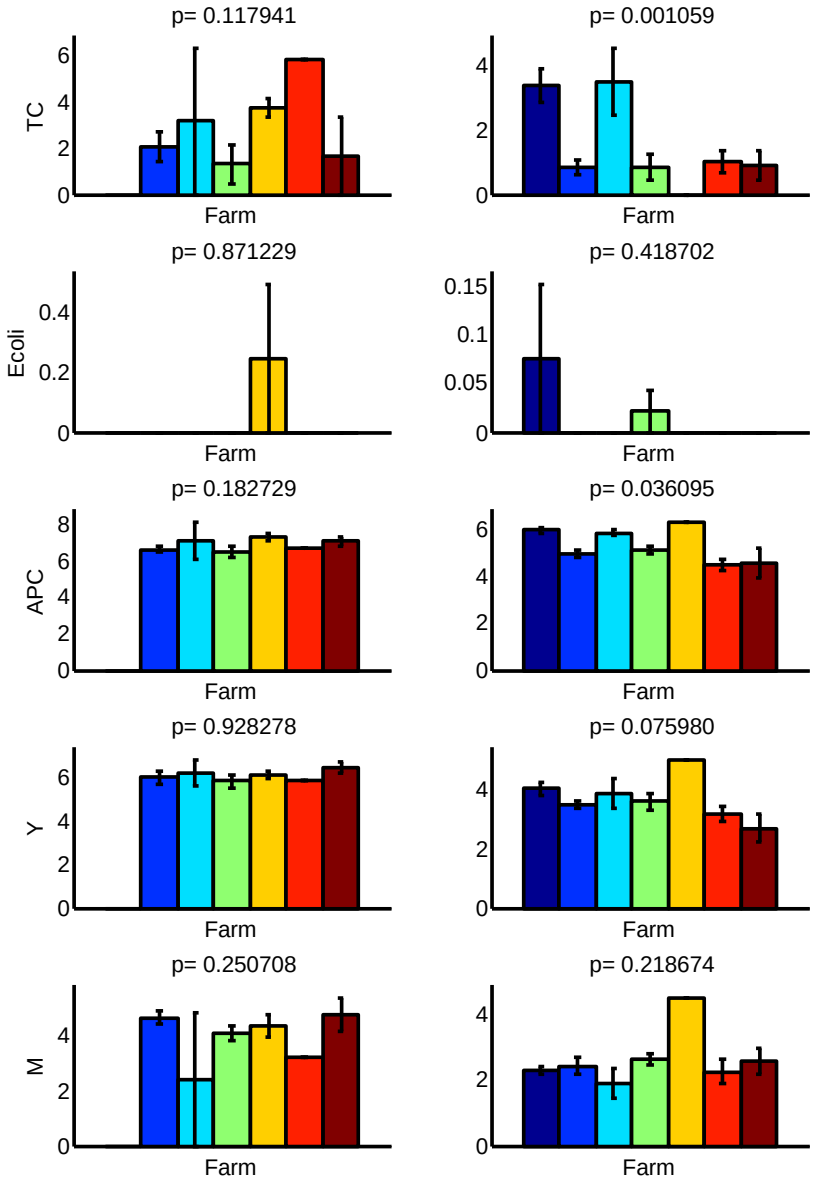

Figure 4.5 Bacteria levels on pre-harvest produce from seven farms(left column is leaf, right column is others)

4.4 Discussion

For pre-harvest samples of different produce types, leafy greens had significantly higher bacterial quantities than other produce types. Comparing pre- and post-harvest (washed

and unwashed) produce samples by different produce types (tomato, leafy green, others), we found that the bacteria quantity is generally higher in post-harvest (washed) than in pre-harvest samples for leafy green samples, whereas levels of indicator microorganisms in pre-harvest tomato samples were higher than in post-harvest (unwashed) tomato samples. We also tested the possibility that irrigation water could act as a potential source of contamination, yet no correlation between the bacterial quantities in irrigation water and in produce was found. Our findings suggest that manual harvesting was not a factor in introducing the tested indicators on produce, which showed in the results for unwashed produce (e.g. tomatoes).

The three food pathogens, *Salmonella*, *L. monocytogenes* and STEC were not observed in the produce samples analyzed. Previous studies have shown that *Salmonella* can be isolated from fresh organic produces, yet the prevalence rate is debatable. [109] reported a prevalence rate of 0.4% for *Salmonella* in pre-harvest organic fruit and vegetable samples collected in Minnesota. Two studies of produce in the retail markets conducted in the United States and in Norway found no positive *Salmonella* samples [110,111]. Similarly, no *E. coli* O157:H7 was detected in a study that included 3,200 organic retail vegetables [112]. Mukherjee et al. did not detect any *E. coli* O157:H7 in pre-harvest organic produce samples [109]. Despite the fact that produce types such as cantaloupe melon, celery and alfafa sprouts have been associated with listeriosis outbreaks in recent years [71], the prevalence of *L. monocytogenes* is generally low [113]. Thus, the lack of pathogen detection in the current study is not surprising considering the relative small number of samples examined and the low prevalence in produce that has been previously reported.

The microbiological safety of post-harvest produces depends not only on growing procedures and environmental factors, but also on handling procedures and produce types. We found that both the percentage of positive samples and the average bacterial quantities were generally higher in pre-harvest tomatoes than in post-harvest (unwashed) tomatoes (Table 4.2 and Figure 3). As for other produce types, bacterial quantities in pre-harvest were higher than post-harvest in terms of APC, Y and M, but lower in terms of TC. The difference between pre- and post-harvest samples of leafy green produce is particularly interesting. Even with washing, bacteria quantities of post-harvest samples were generally higher than those of pre-harvest samples, which suggests common washing procedure fails to reduce bacteria growth on leafy green produce. However, for the one farm that used an EPA approved sanitizer (Tsunami® 100) in washing water, there was no significant difference between pre- and post-harvest samples. Wash water sanitizers are important to prevent the wash water itself from becoming a contaminating step in the processing operation. If the water becomes a reservoir for human pathogens introduced on product contaminated in the field, the wash water sanitizer helps ensure that the pathogens are rapidly killed and cannot contaminate subsequent product [114].

We also observed differences of bacteria quality with respect to produce types. In particular, the bacterial quantities in leafy greens were significantly higher than other produce types in terms of indicator of TC, APC, Yeast and Mold ($p<0.05$). This result is consistent with other studies [109]. It suggests that leafy greens are more susceptible to

contamination and may act as a vehicle of transmission if foodborne pathogens were present.

Production of organic produce typically requires irrigation water during the growing season. There is a growing amount of research elucidating the pathways for produce contamination by water-borne pathogens [115–117]. We thus collected the irrigation water samples and evaluated whether the bacteria indicator quantity on pre-harvest produce is correlated with that in the irrigation water. Although bacteria quantity in almost all water samples satisfied the irrigation water standard, which is below 235 cfu/100 ml for *E. coli* [118], we found the bacteria quantities in surface water is significantly higher than well water in terms of two bacterial indicator microorganisms, generic *E. coli* and TC. However, differences in irrigation water did not cause a significant difference in bacteria quantity on produce, i.e., we observed no significant correlation between the bacteria quantity of irrigation water and produce. This suggests that loss of bacterial quality for produce at the pre-harvest level is likely due to other environmental factors such as soil, temperature, and geographical location, as well as growing procedures such as manure application [119].

In conclusion, during handling stage, washing produce without any sanitizers could produce a potential microbial safety risk on produce in organic farms, although manual harvesting was not a factor in introducing the tested indicators on produce.

Table 4-2 Number of presumptive positive for pathogens

Sample Type	*Salmonella*	*L. monocytogenes*	*stx*$_1$[$] *stx*$_2$[$]	*eae*[$]	Details
Tomato	0	0	0	1	Farm4, pre-harvest
Leafy	0	0	0	1	Swiss Chard, Farm4, pre-harvest
Pepper	0	0	0	1	Farm4, pre-harvest
Other	0	0	0	1	Cucumber, Farm1, pre-harvest
Well water	0	0	0	0	
Surface Water	0	0	0	4	Farm4 (S,M,E); Farm3

*Presumptive positives isolates from Chromagar STEC were recovered. These isolates were all negative for *stx*$_1$ and *stx*$_2$ but positive for *eae* (intimin).

$ *stx*1, *stx*2, *eae* are Shiga toxin *E. coli* (STEC)

Table 4-3 Frequency of positive indicator microorganism detection in produce samples

Indicator	Produce type	Tomato	Leafy Greens	Pepper	Other	Total
E. coli	Pre-harvest	2/23 (8.7%)	1/33 (3%)	0/19 (0)	0/8 (0)	3/83 (3.6%)
	Post-harvest (unwashed)	0/19 (0)	0/1 (0)	0/12 (0)	2/20 (10%)	2/52 (3.8%)
	Post-harvest (washed)	--	0/30 (0)	0/2 (0)	0/10 (0)	0/42 (0)
TC	Pre-harvest	16/23 (70%)	21/33 (63.6%)	8/19 (42.1%)	5/8 (62.5%)	50/83 (60.2%)
	Post-harvest (unwashed)	10/19 (52.6%)	0/1 (0)	8/12 (66.7%)	13/20 (65%)	31/52 (59.6%)
	Post-harvest (washed)	--	26/30 (86.7%)	2/2 (100%)	9/10 (90%)	37/42 (88.1%)
APC	Pre-harvest	23/23 (100%)	33/33 (100%)	19/19 (100%)	8/8 (100%)	83/83 (100%)
	Post-harvest (unwashed)	16/19 (84.2%)	1/1 (100%)	11/12 (91.7%)	19/20 (95%)	47/52 (90.4%)
	Post-harvest (washed)	--	30/30 (100%)	2/2 (100%)	10/10 (100%)	42/42 (100%)
Y	Pre-harvest	23/23 (100%)	33/33 (100%)	18/19 (94.7%)	8/8 (100%)	82/83 (98.8%)
	Post-harvest (unwashed)	17/19 (89.5%)	1/1 (100%)	12/12 (100%)	16/20 (80%)	46/52 (88.5%)
	Post-harvest (washed)	--	30/30 (100%)	2/2 (100%)	7/10 (70%)	39/42 (92.9%)
M	Pre-harvest	22/23 (95.7%)	32/33 (97%)	19/19 (100%)	7/8 (87.5%)	80/83 (96.4%)
	Post-harvest	13/19	1/1	11/12	12/20	37/52

(unwashed)	(68.4%)	(100%)	(91.7%)	(60%)	(71.2%)
Post-harvest (washed)	--	30/30 (100%)	2/2 (100%)	7/10 (70%)	39/42 (92.9%)

Table 4-4 Cultivation and post-harvest practices for farm sampled

Farm	Irrigation water type	Irrigation method	Mulch types of all the produce type	Produce wash water type	Washed produce	Unwashed produce	Irrigation water treatment	Wash water treatment
Farm1 (N=4)	Municipal water	Drip	Plastic: tomato, cucumber	--	--	--	No	No
Farm2 (N=43)	Well water	Drip	Straw: tomato, pepper; Bare: Leafy green, Mizuna, bean, okra, onion; Reusable landscape fabric & bare: summer squash, cucumber, eggplant	Well water	Leafy green, green bean (sometimes), cucumber	Tomato, pepper, squash, eggplant, okra, onion, winter squash	No	No
Farm3 (N=32)	Pond water	Drip	Plastic: tomato, basil, pepper, cucumber	--	--	--	No	No
Farm4 (N=32)	Untreated well or pond water	Drip & overhead sprinkler	Bare: tomato, basil, pepper, leafy green, grape, eggplant, squash, beans Plastic: cucumber	Well water	Leafy green, beans, peppers, eggplant, squash, turnip, cucumber	Tomato, grape (not wash, if produce is clean or recently rained on.)	Drip: pond water filtered to 200 µm (prevent clogging); Overhead sprinkler: pond water not filtered or treated	No
Farm5 (N=34)	Well water	Drip	Straw: tomato; Plastic: pepper, eggplant, cucumber; Bare: leafy	Well water	Squash, eggplant, leafy green	Tomato, pepper, cucumber	No	No
Farm6 (N=26)	Pond water	Drip	Plastic	Well water	Leafy green, onion	Tomato, pepper, eggplant, cucumber, summer squash, zucchini, onion	No	No
Farm7	Municipal water	Drip	Plastic: tomato, pepper, zucchini; Straw: chard; Plastic & straw: cucumber,	Tap water	Chard, mustard, kale, carrot	Tomato, pepper, zucchini, cucumber, squash	No	No

	squash; Bare: mustard, kale, carrot

Chapter 5 Microbiological Assessment of Different Mulches on Epiphytic Indicator Microorganisms on Organic Lettuce

5.1 Introduction

As introduced previously, the bacteriological quality and safety of pre-harvest produce depends on a number of factors during cultivation, such as genetic material, production technology, physiological stage at harvest and agro-ecological conditions. Cropping methods are important factors not only for crop health and yield, but also for food safety risks. A variety of cropping methods are used to grow lettuce on small-scale organic farms in the Mid-Atlantic region of the U.S., but their influence on enteric foodborne pathogens remains largely unexplored. There is an increasing amount of information on the potential of in-field contamination from direct sources such as manure [43] and irrigation water [41]. Assessment of the risk of contamination requires not only the identification of the sources of contamination, but also the impact of cultivation practices. This includes determination of the impact of cropping methods on fate and persistence of pathogens and indicator microorganisms pertinent to food safety.

Mulching is a widely used cropping approach to enhance the growth of fresh produce. A variety of materials are used as mulches, including paper, plastic, straw and other organic materials. Mulches are applied to the soil surface at various times of the year for different purposes. At the beginning of the growing season, mulches are often applied to avoid heat loss during the night, which allows early seeding and encourages fast growth of transplanted crops [120]. In temperate climates, mulches are often used to protect plants from cold and suppress growth in winter and early spring to prevent freeze thaw damage

[121]. In the summer, organic mulches are usually applied to hold moisture and prevent high-temperature, and to control weed growth. In addition to regulation of soil temperature, mulches also have complex effects on soil moisture. It can reduce evaporation by preventing sunlight from the soil surface, and can also prevent water from reaching the soil by absorption and block rainwater from penetrating soil. A number of studies showed the effectiveness of mulch on increasing the yield of a variety of crops [95,97,122–127].

Among all the mulches for growing produce, the use of plastic mulch is the most common. Black polyethylene is the most popular type due to its benefits and low cost. However, in the last few years, the use of starch-based biodegradable films (mainly from corn, potato and rice crops) has been introduced as an alternative to conventional mulches to reduce the environmental impact. When these mulches are placed in contact with suitable soil moistures and microorganisms [128], they are biodegraded by soil microorganisms at the end of the crop season. Paper mulch is less common but can also be decomposed [129]. Other mulches, such as straw, hay and compost are used in farms as well for different produce types. A study in Virginia showed organic mulches reduced temperature and maintained higher soil moisture levels than others, such as black plastic mulch [129].

Mulching brings many benefits, such as retention of soil moisture, regulation of soil temperature and suppression of weed growth, and can lead to increased crop yield [13097]. Mulching had significant effects on the lettuce's head, leaf and stem growth, and total yield compared with bare ground [95]. Plastic mulches can directly affect the plant micro-

climate by decreasing soil water loss and modifying the surface radiation budget [96], thus providing high productivity and increased nutrient accumulation in lettuce [97].

Despite these benefits, there are few studies on the effects of mulching on soil and plant microbiota or specifically the survival of foodborne pathogens on produce. Mulches may affect plant pathogens as well as food pathogens. Different types of mulches have been reported to have different effects on plant pathogen risk. For example, one study showed that straw mulch reduced levels of center rot caused by the bacterium *Pantoea ananatis* on sweet onion, while black plastic mulches had the opposite effect, speeding up the onset of the epidemic [98]. Grass mulching reduced the production of apothecia of *Sclerotinia sclerotiorum* in soils rich in organic matter [131], However, there are few studies investigating the effects of mulches on the crop microbiome. Plastic mulch was reported to result in higher initial counts including coliforms, yeast and mold, mesophilic, psychrotrophic and lactic acid bacteria (LAB) before storage [132]. The effect on microbiological risk could also vary with time, particularly for organic mulches which could have long-term effects on the soil bacterial communities [22]. Studies on microbial food safety risks of different mulches or the fate and dispersal of foodborne pathogens are even scarcer. In a study of splash dispersal of *Salmonella* during a simulated rain event indicated that plastic mulch appeared to enhance the dispersal of *Salmonella* to tomatoes [133].

The objective of this study was to assess the impact of different mulching methods – polyethylene plastic, biodegradable plastic, paper and bare ground as a control – on the survival of epiphytically-associated fecal indicator bacteria on organically grown lettuce

over time during two different cultivation seasons (spring and fall). Three indicator microorganisms were tested, *E. coli*, *Enterococcus* spp. and fecal coliforms. This study provides novel data on the effect of using various mulches on the survival of bacteria that can indicate the safety of leafy greens. A better understanding of the impact of cropping methods on the fate of epiphytic enteric bacteria on leafy greens provides an important step in determining cropping practices for the best food safety outcomes.

5.2. Materials and Methods

Field sites and plots design.

The field experiment was conducted at the Wye Research and Education Center (Wye REC) of the University of Maryland, Queenstown, MD. Experiments were conducted during the spring and fall seasons of 2013.

There were four treatments of different mulches: bare ground (BG)(no mulch control), black polyethylene plastic (PP), corn-based plastic (biodegradable) (CP) and paper mulch (PM). Plots were arranged in a randomized complete block design with four replications per treatment in the spring and three or four replications and one control (without inoculation) in the fall. Each treatment consisted of three or four double-row beds of size 15 feet by 2 feet, with 4 feet spacing between the beds to avoid drift and cross contamination. The plot layout is shown in Fig. 5.1 for both seasons. A buffer row with polyethylene plastic mulch and planted with lettuce was laid on the right to separate the research plot from other research fields. There were no research fields to the left of the plot. Twelve lettuce heads were planted 1 foot apart in rows in each bed.

The soil at Wye was a loam soil with a pH of 5.8 to 6.2. Soils in the plot area were chisel plowed. Raised planting beds were formed with a rototiller and bed shaper. The plots used drip irrigation and irrigated with well water. Drip irrigation T-tape,8 ml thick, with 12 inch emitter spacing, 0.45 GPM per 100 ft tape @ 12 PSI was buried 1-2 inches deep. One line of drip tape was used per bed and was placed midway between two rows of plants. Pelletized chicken manure pellets (Purdue) were applied at a rate of 3000 lbs per acre.

Lettuce cultivation

Romaine lettuce cultivar Parris Island Cos seeds were started in the greenhouse in 1" pots (April or September) and transplanted in the field (Wye REC) 3 weeks post germination. Manure application to lettuce was performed 8 weeks post transplantation, as described below.

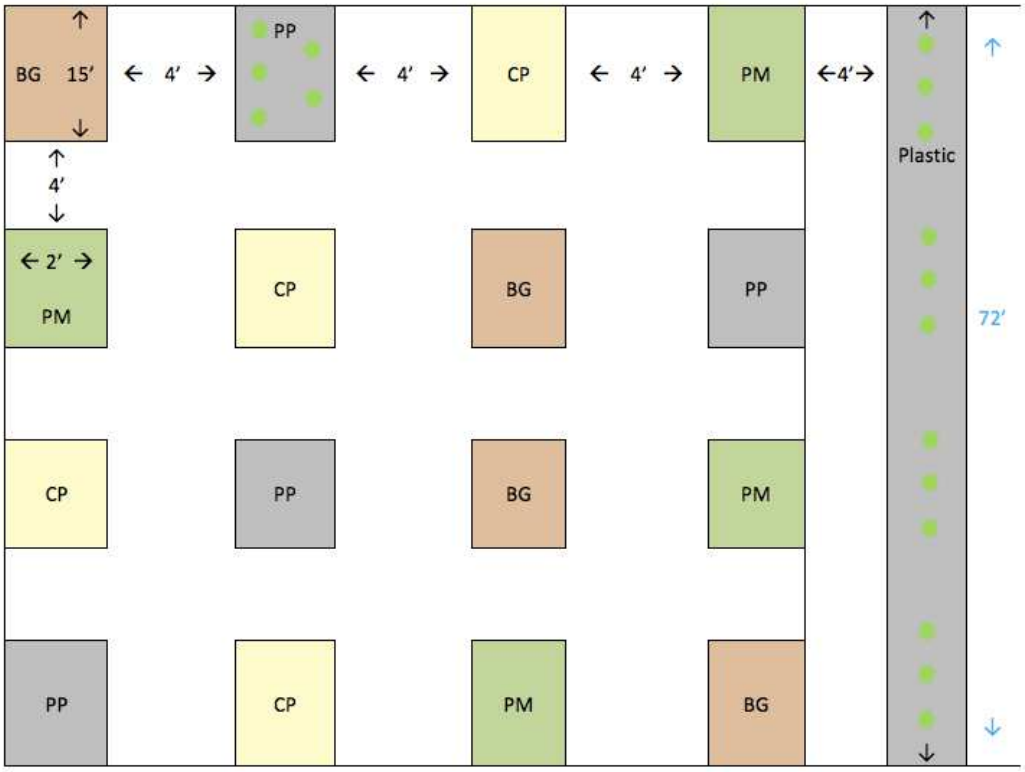

Figure 5.1 The randomized complete block design of the field plot (fall season). The plot design for spring season was similar, excluding the bottom row. Different mulches are labeled with different colors. Three types of mulch were investigated - polyethylene plastic (PP), corn-based plastic (CP), paper mulch (PM). Bare ground (BG) served as a no-mulch control. Each block was planted with a row of twelve lettuce heads.

Bare Ground (BG)

Paper Mulch (PM)

Corn-based plastic (CP)

Polyethylene Plastic (PP)

Figure 5.2 Top: Overview of the experimental plot. Bottom: Photographs of the four treatments considered in this study.

Manure Collection and Inoculum Preparation

Liquid dairy manure was collected fresh from the Clarksville Research and Education Center, Clarksville, MD of the University of Maryland, 2 days before inoculation day. Non-pathogenic *E. coli*, previously isolated from liquid manure from the same site, was cultured in Tryptic Soy Broth (TSB) (BD, France) for 24 h at 37°C to a concentration of 8 log cfu/ml and 20 ml broth added to the manure. The manure was enumerated for *E. coli*, *Enterococcus* and fecal coliforms before and after supplemented with *E.coli* as well as on the morning of inoculation day. From the manure, dilutions were made of 1:5 (original dilution), 1:50 and 1:500 using PBS and 100 µl of each dilution was spread on Tryptone Bile Glucuronic Agar (TBX) plates (HiMedia Laboratories Pvt. Ltd, India) for *E. coli* , Enterococcosel Agar plates (EA) (BD, Germany) for *Enterococcus* and m-FC agar plates (Criterion, CA) for fecal coliforms. The plates were incubated at 44°C for 24-48 h (TBX), 37°C for 24-48h (EA), and 44°C for 24 h (m-FC) and plates counted.

E. coli counts in spring and fall were $4.7*10^7$ cfu/ml and $1.6*10^4$ cfu/ml; *Enterococcus spp.* count was 1700 cfu/ml in spring and the fecal coliforms counts in the spring and fall were $5*10^7$ cfu/ml(spring) and 4,400 cfu/ml (fall).

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Table 5-1 Bacterial concentrations in manure.

	***Enterococcus* spp.**	***E. coli***	**Fecal coliforms**
Spring	$1.7*10^3$ cfu/ml	$4.7*10^7$ cfu/ml	$5*10^7$ cfu/ml
Fall	Not measured	$1.6*10^4$ cfu/ml	$4.4*10^3$ cfu/ml

Inoculum application and sampling

A 100-ml sterile specimen cup container was used to inoculate the contaminated manure to the lettuce plant. A colander was placed over the lettuce heads to disperse the inoculum more evenly over the plant. A 100 ml aliquot of the inoculum was applied in this manner to each lettuce, taking care not to sprinkle any inoculum away from the lettuce.

Lettuce and soil samples were collected prior to inoculation, and 30 min after inoculation. Thereafter, samples were taken at 1, 3, 5, 7, 10 and 14 days after inoculation. During sampling, 2-4 leaves of lettuce were harvested randomly in each bed. The leaves from the inner and middle layers of the plant were collected with sterile scissors and collected into a labeled sterile Ziploc bag. At the same time, 200 g soil samples from the surface to 10 cm deep from each bed were sampled using scoops into a labeled sterile Ziploc bag. The samples were temporarily stored in a cooler during transportation to the lab located at the Department of Plant Science and Landscape Architecture of University of Maryland (60 miles from the research field), and subsequently refrigerated until analyzed.

Sample processing

Samples were processed within 48 hours. For each sample, 20 g of leaves were weighed from each sample into a sterile whirlpak bag. Eighty millilitres phosphate-buffered saline (PBS) were added to each bag and stomached with a laboratory stomacher (Seward, Stomacher 400 circulator, U.K.) for 2 min at 250 pulses per min at room temperature. From the washate, dilutions were made of 1:50 and 1:500 using PBS, and 100 µl of original washate (1:5) and each prepared dilution were spread on TBX, EA, and m-FC

agar plates (single plates). The plates were incubated at 44°C for 24-48 h (TBX), 37°C for 24-48h (EA), and 44°C for 24 h (m-FC) and plates counted.

Ten-gram soil samples were weighed from each composite sample into a sterile whirlpak bag. To each bag, 90 ml of PBS were added and shaken in a shaker for 2 mins. From the washate, a 1:100 dilution was made using PBS and 100 µl of each dilution were spread on TBX, EA and m-FC agar plates. The plates were incubated at 44°C for 24-48 h (TBX), 37°C for 24-48 h (EA) and 44°C for 24 h (m-FC) and plates counted.

Daily amounts of rain (cm) and high and low temperature (°C) were obtained from Wye REC weather station database.

Data analysis

The software Matlab (R2013a, Mathworks) and JMP® Pro 10.0.2 were used for statistical treatments. A logarithm transformation were performed on the raw data using the formula $\log_{10}(1+c)$, where c is the measured concentration in the unit of cfu/g. Quantile-quantile plots were used to test the normality of the data. A pair-wise Wilcoxon signed rank test was used to test the difference across three types of mulches and bare ground. Decline rates were calculated using linear regression with data between day 0+ (after inoculation) and the last data with indicator bacteria detected (>0 log cfu/ml).

5.3. Results

5.3.1. Assessment of Progression of Bacterial Counts in Soil and on Lettuce Leaves over Time at Different Seasons.

E. coli

Leaves: The progression of generic *E. coli* counts over time is shown in Fig. 5.3. *E. coli* was not detected in any of the leaf or soil samples before inoculation (day 0-). After inoculation, there was a drastic increase of *E. coli* counts (day 0+) on the leaf samples (from 0 log cfu/g to 5.1 log cfu/g), which then decreased over time. Although the initial *E. coli* concentration in manure was quite different between the fall and spring experiments, the decline rates were similar in both seasons (-0.58 log cfu/g/day in fall and -0.55 log cfu/g/day in spring, averaged across all samples). *E. coli* was not detected after day 5 in the fall experiment, but was still present in the spring experiment even on day 14. There are no significant differences in decline rates among mulch types.

Soil: The changes in *E. coli* counts in soil samples progressed slower than in the leaves. The peak bacterial counts occurred three days after inoculation, and then decreased over time. At 14 days after inoculation, the average concentration of *E. coli* was 0.49 log cfu/ml (spring) and 0.17 log cfu/ml (fall), which are higher than the concentrations in the leaf samples (0.13 log cfu/ml, spring; 0 log cfu/ml fall).

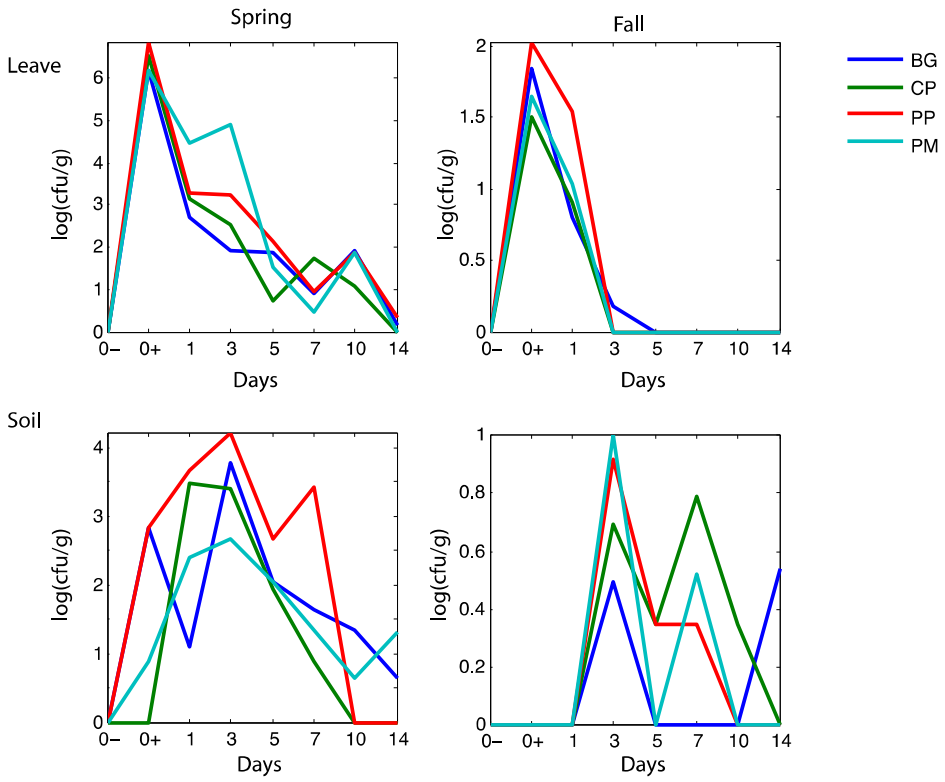


Figure 5.3 Progression of *E. coli* counts on lettuce leaves (top) and in the soil (bottom). Each trace represents samples using different mulch. Blue: bare ground; green: corn-based plastic; red: polyethylene plastic, cyan: paper mulch.

Fecal coliforms

Leaves: The change of fecal coliform counts in soil and on lettuce leaves are shown in Fig. 5.4. No fecal coliforms were detected on the leaves before inoculation (0-) in both seasons, regardless of mulch type. For the spring experiment, there was a dramatic increase of fecal coliform counts on the leaf samples right after inoculation (day 0+), from non-detectable before inoculation to 6.73 log cfu/ml, which then gradually decayed overtime (-0.61 log cfu/ml/day). For the fall experiment, an increase of fecal coliform counts was observed on the leaves after inoculation. However, the peak bacterial

count happened three days after inoculation. There was a resurgence of fecal coliform counts at day 10.

Soil: Unlike the leaf samples, fecal coliforms were present in the soil before inoculation in the fall, but were not detected in the soil in the spring. Similar to the *E. coli* counts, the progression of fecal coliforms counts in the soil occurred slower than on the leaves. The peak happened one day after inoculation in the spring and seven days after inoculation in the fall. The concentration after 14 days post-inoculation was 0.38 log cfu/ml (spring) and 1.47 log cfu/ml (fall).

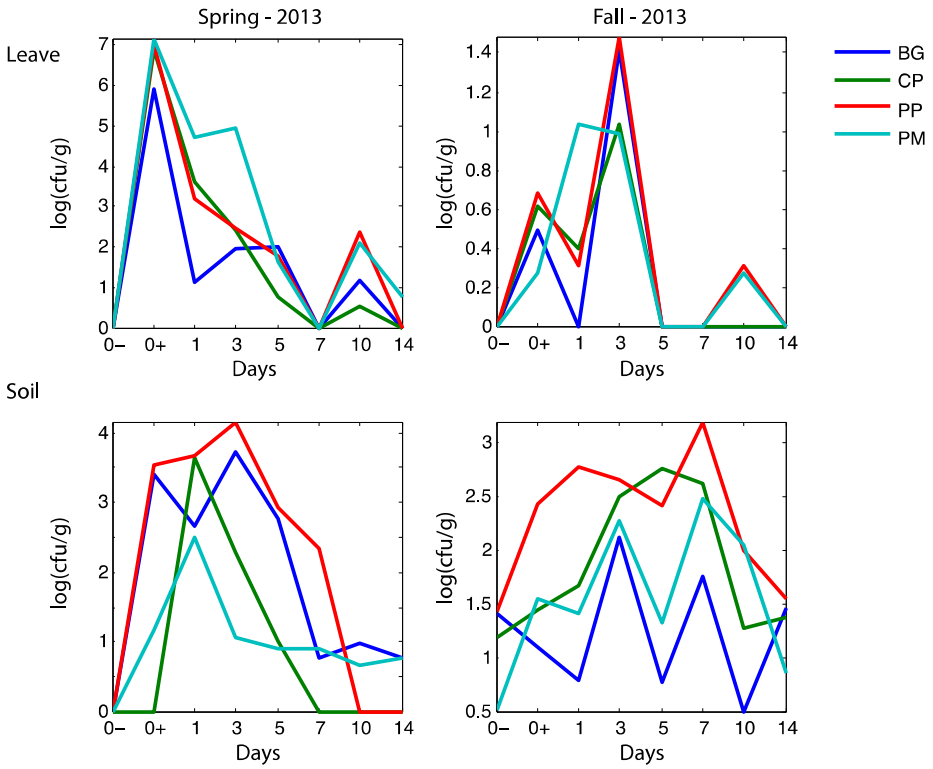


Figure 5.4 Progression of fecal coliform counts on the leaves (top) and in the soil (bottom). Each trace represents samples using different mulch. Blue: bare ground; green: corn- based plastic; red: polyethylene plastic, cyan: paper mulch.

Enterococcus spp.

Leaves: The progression of *Enterococcus* spp. concentration over time is shown in Fig. 5.5. *Enterococcus* was detected at low concentrations on day 0-, prior to inoculation (mean=2.26 log(cfu/g) in spring, 0.13 log(cfu/g) in fall) . There was no clear trend for *Enterococcus* spp. survival on leaves over time.

Soil: In contrast to *E. coli and* fecal coliforms, in both seasons, *Enterococcus* spp. counts in soil samples increased gradually after inoculation. The maximum bacterial counts occurred 14 days after inoculation. No further samples were taken following the termination of the experiment at day 14.

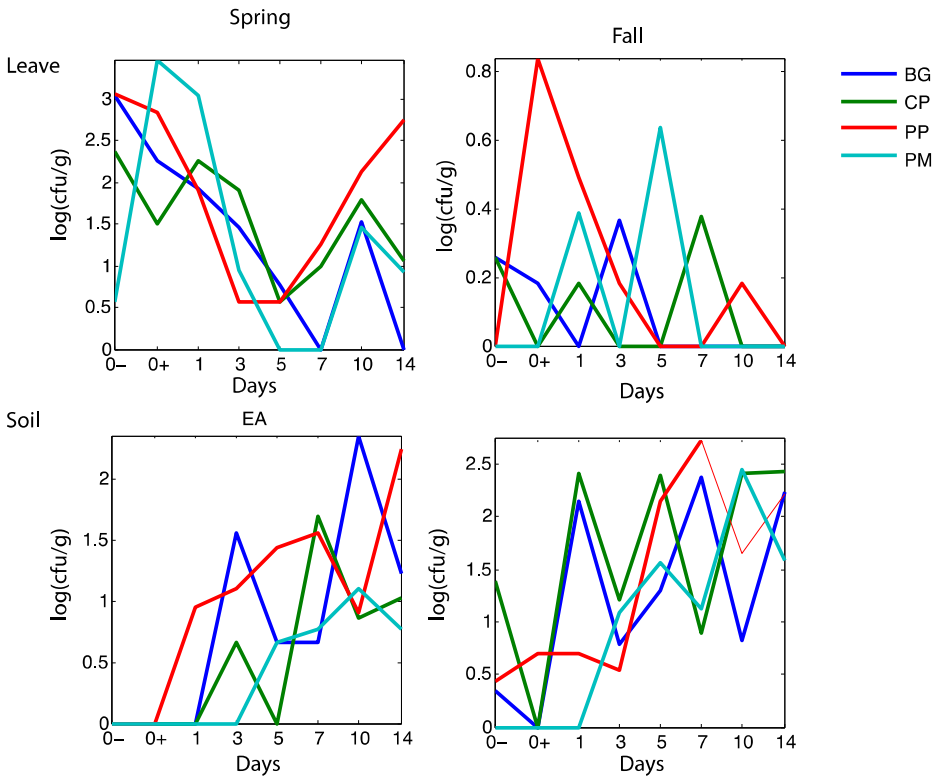


Figure 5.5 Progression of *Enterococcus* spp. on lettuce (top) and in the soil (bottom). Each trace represents samples using different mulches. Blue: bare ground; green: corn-based plastic; red: polyethylene plastic, cyan: paper mulch.

5.3.2. Difference across Mulches.

The major goal of this study was to investigate how the survival of fecal indicator bacteria varies in response to different mulches. To achieve this goal, it was necessary to compare bacterial counts for different mulches. However, factors other than ground cover appeared to impact bacterial counts. Bacterial concentrations were expected to be

strongly dependent on the time since inoculation, as well as other factors such as temperature and precipitation.

One common approach to remove the impact of these additional factors is to use ANOVA with repeated measures, which implicitly assumes data obeys normal distribution. This assumption was checked by comparing the sample quantiles with theoretical quantiles from a normal distribution (Fig. 5.6). However, none of the data appeared to follow the prediction of the normal distribution (red dashed line) in the quantile-quantile plots below.

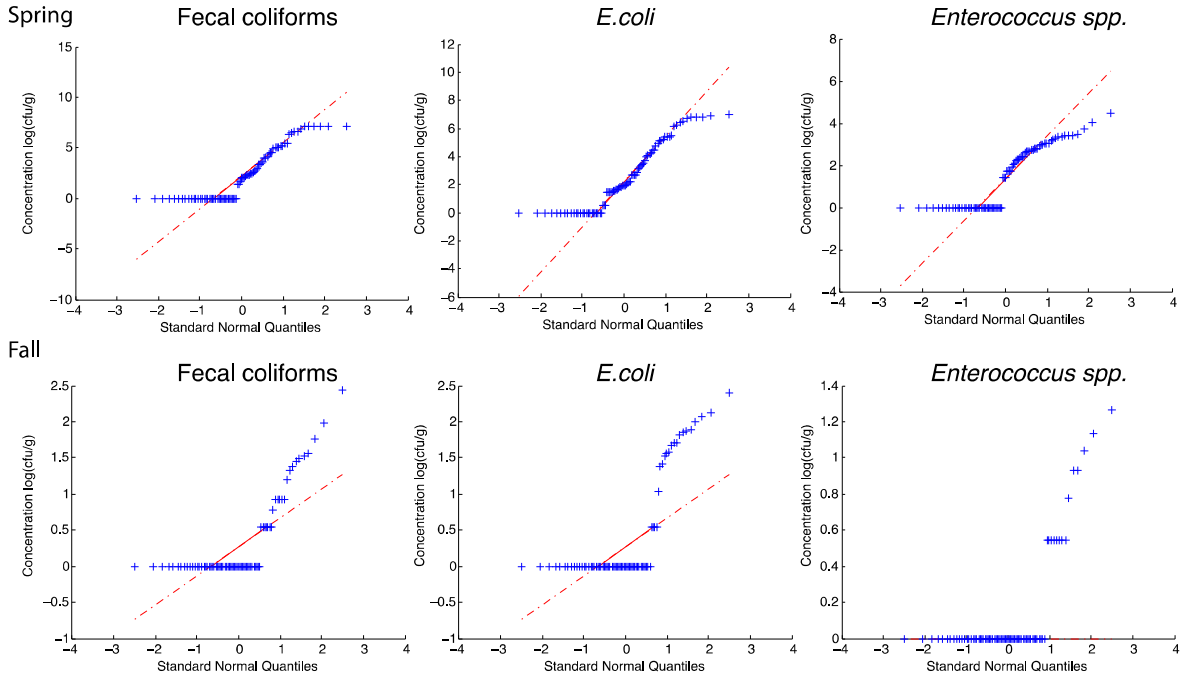


Figure 5.6 Quantile-Quantile plots of sample quantiles of three indicator bacterial counts on the leaf samples versus the theoretical quantiles from a normal distribution. The red dashed line indicates prediction of the normal distribution. The top row displays spring season data, bottom row displays fall season data.

A non-parametric test was used to reveal differences due to ground cover. The null hypothesis was that there are no differences among samples collected at the same time with different ground covers (BG, CP, PP and PM). The alternative hypothesis was that there are differences of among samples collected at the same post-inoculation time that were due to mulch differences. In other words, the effects of other factors (temperature, precipitation, lettuce leaf exudates) are assumed to be identical and more influential for samples collected at the same time.

Samples from two mulches were compared in a pair-wise fashion. For instance, there were three samples for the four mulch types (BG, PP, CP, PM) at each post-inoculation sampling time for the spring season, which then leads to nine pairs for comparison for every two mulch types. Across time, there can be as many as 8 days × 9 pairs = 72 pairs of comparisons. The Wilcoxon signed rank test was used on the pair-wise comparisons to test whether there were significant differences between two types of ground covers. How often one mulch had higher bacterial counts than another mulch was also reported, which reflects the magnitude of difference between two mulches.

The result of this analysis is shown in Fig. 5.7 for lettuce and in Fig. 5.8 for soil. For the lettuce samples, the mulch differences are largely consistent across seasons (top versus bottom row), despite different magnitudes. This similarity is manifested in the fecal coliform counts, where BG<PP<CP<PM for both seasons. For both *E. coli* and *Enterococcus* spp. counts, relationships BG<CP, PP<CP and PM<CP was obtained in both seasons.

On the other hand, the differences across mulches for the soil samples were more variable across seasons (Fig. 5.8). There is almost no relationship that was observed for both seasons.

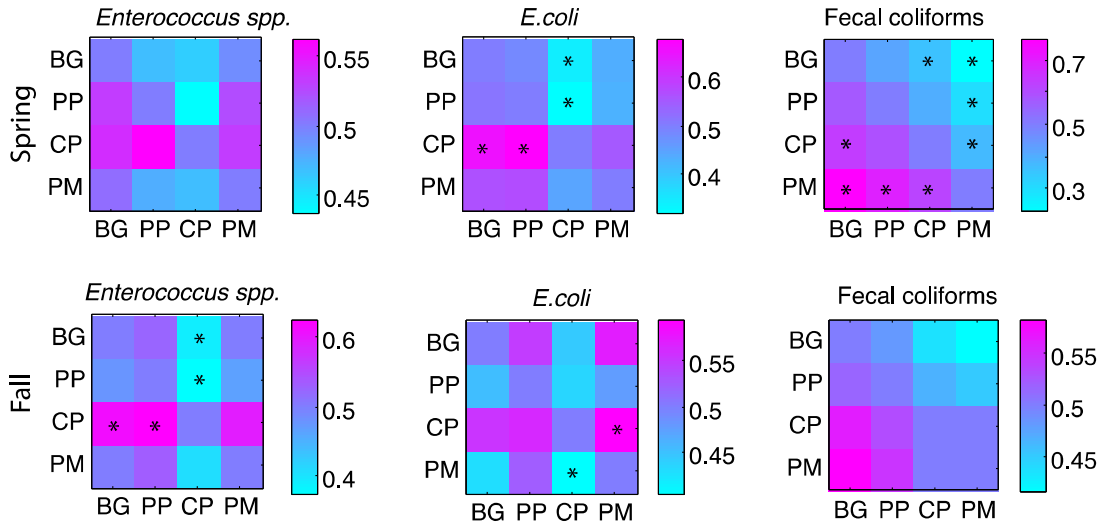


Figure 5.7 Comparison of different mulches on the survival of three indicator bacteria, *Enterococcus* spp. (left), *E. coli* (middle), Fecal coliforms (right) on the leaf samples for the spring season (top) and for the fall season (bottom). Each square represents fraction when mulch type on the right has higher counts than mulch type on the bottom. * $p<0.05$, Wilcoxon signed rank test.

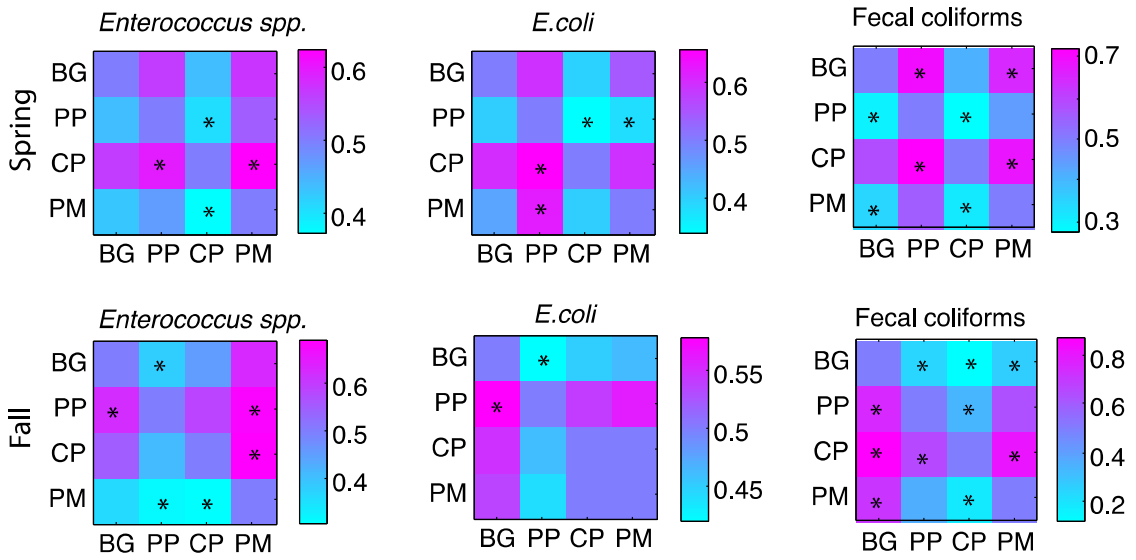


Figure 5.8 Comparison of different mulches on the survival of three indicator bacteria, *Enterococcus* spp. (left), *E. coli* (middle), Fecal coliforms (right) on the soil samples for the spring season (top) and for the fall season (bottom). The cells with *are those where the level of indicator microorganisms observed with the different mulches were significantly different ($p<0.05$, Wilcoxon signed rank test).

5.4 Discussion

Despite the benefits of using mulch for water conservation, temperature regulation and weed suppression, little is known about the effect of mulching on the survival and growth of foodborne pathogens. The goal of this study was to fill in this gap by comparing the survival of three indicator bacteria for foodborne and waterborne pathogens, *E. coli*, fecal coliforms, *Enterococcus* spp., with various types of mulching. Consistent differences across mulch types were observed in terms of survival of indicator bacteria on the leaf samples, and season-dependent difference in the survival of soil samples. These data could support a more thorough risk assessment of the effect of mulching on pathogen survival.

5.4.1 Use of Mulches in Agriculture

The temperature under polyethylene mulches has been reported to be higher than biodegradable mulches [128]. This can be advantagus in cool weather and disadvantagous in hot weather, and could account for higher bacterial counts in the fall and lower counts in the spring under corn-based plastic and other mulches in the study. An early study showed that paper mulch could reduce afternoon soil temperature and organic mulches reduced afternoon soil temperature and maintained higher soil moisture levels than other treatments The higher fecal coliforms count under paper and corn-based plastic mulch could be attributed to this. The higher decline rate in the fall season could also be related to the lower temperature during the experiment (Fig. 5.9). The added carbon source provided by paper and biodegradable plastic could also possibly contribute to higher bacterial counts.

5.4.2 Effect of Mulching on Microbial Activity and Community Structure

Organic mulches serve as food for many microorganisms in the soil. By regulating the temperature and moisture of the soil, activity of many microorganisms can continue at an even rate. Mulching likely increases the activity of many microorganisms in the soil, and may reshape the bacterial community structure. One study showed that organic mulch has long-term effects on the soil microbial activity and community structure in the top few centimeters of the soil profile [22], and the effect is dependent on mulch types. Another study showed plastic mulch could affect the *Salmonella* dispersal with 10 minutes of rain, and 0 to 10 minutes of rain helped *Salmonella* dispersal [133]**.** In this study, the bacterial

count increased after rain, especially for the lettuce under black plastic mulch, which is consistent with the former study. Another study found that a significant increase in the fecal coliform numbers may be associated with average rainfall amounts [134]. That could explain a resurgence of fecal coliform counts at day 10, which was likely due to the precipitation at the same time (Fig. 5.9). The change of fecal coliform counts in the soil samples progressed at a slower rate than the leaf samples, presumably due to a transfer delay from leafs to the soil.

5.4.3 Effect of Mulching on Survival of Foodborne Pathogens

There are very few studies investigating the effect of mulches on survival of foodborne pathogens. Available evidence suggests the effect on the microbiological risk depends on mulch types and may vary between soil samples and crop samples (*40*, *79*)[131]. In our study, we compare the effect of three different mulches and bare ground treatment on the survival of indicator bacteria both on the lettuce phyllosphere and in the soil. Consistent with previous studies, we found the effect depends on mulch types and differs for leaf samples and soil samples.

To our knowledge this work represents the first study comparing the effect of several mulches used in organic leafy greens production on fecal indicator fate and persistence. Only three types of indicators were assessed, and the utility of *Enterococcus* spp. in the phyllosphere was found to be inadequate, due to this taxon being found naturally prevalent in the lettuce phyllosphere. To minimize introduction of bacterial indicator species due to wildlife, an electric fence was installed around the field to deter wild

animals. In spite of limitations, this data is useful to design more rigorous and targeted studies to assess the impact of mulching on pre-harvest produce safety. These types of scientific data can help farmers evaluate the most adequate mulch practices to adopt for the best food safety outcomes when growing leafy greens. Although no immediate risks are apparent, bacterial persistence in soil could affect subsequent crops. Many more studies could be done based on this study, for instance, the analysis of bacterial communities in the lettuce and soil under different mulches. Due to the fact that bacterial indicators have a limited role to evaluate all the bacteria species and their interactions and microbial communities in the soil are very complex and diverse, more work on analysis of bacterial communities in produce and soil need to be explored to understand better the effects of the mulches on the microbial safety of produce.

Chapter 6 Assessment of the Impact of Different Washing Methods on Epiphytic Bacteria Communities on Organic Lettuce

6.1 Introduction

Organic produce differs from conventional produce not only during the growing process, but also during post-harvest handling. A variety of conventional post-harvest decontamination methods, such as irradiation, chemical washes, and synthetic disinfectants, are prohibited in organic farming. Other practices, such as the use of chlorinated water are only optional and are not widely adopted by organic farmers. In Maryland, most small to medium scale farmers minimally process most fresh produce crops post-harvest, either not washing the produce, or washing the produce without the addition of sanitizer to wash water (MOFFA survey 2012). The influence of post-harvest handling processes, or lack thereof, especially in washing methods, on the microbiological safety of organic produce has not been fully investigated.

Sanitizers are typically added to wash water to minimize cross-contamination. The effectiveness of chlorine and other commercial sanitizer agents (e.g., Tsunami 100) in reducing epiphytic microorganisms is questionable. One study showed that although washing solutions were more effective in reducing the microbial load than water alone initially, the difference diminished after 8 days of storage simulating a commercial shelf-life [99]. It is clear that epiphytic bacteria are able to grow even under low storage temperatures.

Despite the similarity in indicator bacteria load after 8 days of storage, the risk of foodborne pathogen contamination could differ. It remains unknown how different washing method change the epiphytic bacteria community composition and structure. A number of studies have revealed complex, yet significant interactions between foodborne pathogens such as, *E. coli* O157:H7 and *Salmonella*, and other species of epiphytic bacteria. For example, several species of phytobacteria, such as *Pseudomonas syringae* [80], *Pseudomonas fluorescens* 2-79 [81,82] and *Enterobacter asburiae* [84], can significantly reduce the growth of human enteric pathogens, while other species of phytobacteria, especially phytopathogens, can promote the attachment and growth of human pathogens [79,84,85].

The goal of this study was to investigate whether different washing methods changed the indicator bacterial counts as well as the epiphytic bacterial community structure on lettuce. Indicator microorganisms were quantified by culture methods, and bacterial communities were compared using a bacterial fingerprinting method, to identify differences in washed and unwashed lettuce following harvest and handling, both immediately after washing and after 5 days of low temperature storage. Automated Ribosomal Intergenic Spacer Analysis (ARISA) was used for fingerprinting lettuce phyllobacterial communities. ARISA is a useful, rapid and cost-effective way to compare microbial community profiles that is widely used and found to be comparable to Next-Generation DNA sequencing methods in identifying community patterns and differences [136]. Findings indicate that although reductions in indicator bacterial counts obtained after washing diminished after storage, differences in the ARISA profiles

persisted. This study indicates that different washing treatments have long-lasting effects on the epiphytical bacterial communities. Such impacts could result in different microbial risks through interactions between foodborne pathogens and other species of epiphytic bacteria.

6.2 Materials and Methods

Study sites and sample collection.

Lettuce samples were collected twice from an organic farm in Maryland in April 2014. Samples were collected in sterile Whirlpak bags, using gloved hands. Gloves were sterilized with alcohol in between sample collections. Bags were placed immediately on ice. At the farm, 600 g unwashed lettuce samples, harvested by farm personnel on sampling day, and 200 g washed lettuce samples, by farm personnel on sampling day using well water and Tsunami 100 (an EPA-registered product approved as a sanitizer for produce wash water), were collected.

Sample processing

Unwashed lettuce samples were separated into three batches of 200 g each for three treatments with four replicates (25 g per replicate sample) and two storage times. Nothing was done to one of the treatments. The other two treatments were washing in 2 L tap water or 100 ppm sodium Hypochlorite (bleach) in a lettuce wash container, submerged and washed for 2 min and then placed into 8 bags (25 g for each). For each treatment, four bags of samples were processed at day 0 immediately and another stored

at 4°C for 5 days for later processing. As for washed lettuce, one of them was processed immediately, and another kept in the fridge for 4°C for 5 days then processed (Table 6.1).

Table 6-1 Washing treatments of lettuce, with 4 replicates of each condition

Sample Type	**Activity**	
Unwashed	25g process day 0	25g store 4°C and process after 5 days
Unwashed	25 g treat with tap water and process day 0	25 g treat with tap water at day 0, store 4°C and process after 5 days
Unwashed	25 g treat with bleach and process day 0	25 g treat with bleach at day 0 , store 4°C and process after 5 days
Washed (PAA, by farm)	25 g process day 0	25 g store 4°C and after 5 days

Processing Day 0 Samples

To each bag containing 25 g of lettuce leaves, 225 ml of sterile 0.1% peptone water (BD, France) were added. Bags were placed on a shaker (200 rpm) (VWR) for 1 min, and bags turned and shaken for another min. Samples were sonicated (Branson Sonicator) at high speed for 2 min and then shaken again for another 2 min, turning bag half way through. The wash rinses of the samples were collected and appropriate dilutions made in 0.1% peptone water. One ml of each dilution was placed on 3M petrifilms (TC/ *E.coli*: 10^{-1}-10^{-3}, APC: 10^{-3}-10^{-4}, Y&M: 10^{-2}-10^{-4}) and incubated at 37°C for 24-48h (depending on petrifilms) and plates read as per manufacturer's recommendations. The remaining rinse solution was transferred to 50 ml centrifuge tubes and centrifuge at 8,000 rpm for 10 min. After centrifugation, the solutions were decanted and more rinse added for centrifugation until all rinsate for a sample was processed. After the last centrifugation, the supernatant

was decanted and the pellets were pipetted into a sterile, labeled, microcentrifuge tube (2 tubes for 1 sample) and frozen at -20°C.

The process steps were the same as Day 0 for the samples stored at 4°C for 5 days. The pellets were used for DNA isolation, purification and bacterial community fingerprinting.

DNA isolation and quantification

DNA was extracted by using an UltraClean Microbial DNA Isolation Kit (Invitrogen, Germany). The DNA samples are quantified with NanoDrop-1000 Spectrophotometer to check for quantity and purity.

Bacterial Automated Ribosomal Intergenic Spacer Analysis (ARISA)

The four replicate DNA extracts for each of the 16 samples (n = 64) were titrated to a standard working concentration (10 ng μL^{-1}). The DNA was amplified by PCR targeting the intergenic spacer region (ISR) region between 16S and 23 S rRNA genes with the universal primers 1392f; 5′ - GYACACACCGCCCGT - 3′ and a 5′FAM labelled 23Sr 5′ - GGGTTBCCCCATTCRG - 3′ (Fisher & Triplett, 1999; Hewson & Fuhrman, 2004). The 25 μL reaction contained 0.5 μM of each primer, 1 unit of Taq polymerase (Invitrogen), 1× buffer with 2.5 mM $MgCl_2$ (Life Science), 0.25 mM of each dNTP (New England BioLabs Inc).The reaction was held at 94 °C for 2 min followed by 30 cycles of amplification at 94 °C for 30 s, 55 °C for 30 s and 72 °C for 90 s, with a final extension at 72 °C for 5 min (Slabbert et al., 2010). The PCR products were run on 1% agarose gels

for confirmation of PCR product. Two replicates did not work due to low DNA quality. The total number was 62 for ARISA. The 62 replicates were purified by PureLink Quick PCR Purification Kit (Invitrogen, Germany) for purification of PCR products. ISR separation was performed at University of Maryland Genomics Core, on an Applied Biosystems 3730xl Fragment Analyser (Life Technologies) in 96 well plates. Each well contained 4 µl of sample, 1µl GeneScan 1000ROX standard and 5 µl HiDi Formamide. Electropherograms were subsequently interpreted via Genemapper software Version 4.0 (Life Technologies).

Data analysis:

Data generated by GeneMapper v.4.0 included peaks measured in base pairs (bp) and the area of each peak. Peaks falling between 80-600 bp were considered as Operation Taxonomic Units (OTU) with a resolution of 1bp. The 80-600 bp range was based on accuracy of the size detection standard. Area data from each sample were standardized by calculating the percentage area of each peak relative to the total area of all peaks within that sample. Data from all samples were then manually checked to ensure accurate peak binning. The presence and absence and relative area data were subjected to statistical analysis.

Data from ARISA was imported into PRIMER 6 (Plymouth Routines in Multivariate Ecological Research-E - version 6.1.15) from PRIMER-E Ltd., Plymouth, UK, a statistical software package for the analysis of ecological, multivariate data. Bray-Curtis method was used to form similarity matrices for the bacteria community. Two separate

similarity matrices were calculated from ARISA data, one from presence-absence data and the other from relative area values. The similarity matrices were also used in non-metric Multi-Dimensional Scaling (MDS) ordination which represent the relationship among samples. Two near points represent more similarity to each other than others located at a distance. The non-parametric permutation procedure ANOSIM (analysis of similarity) was used for significance testing of sample data, which combines MDS ranking similarity with Monte Carlo randomization to form significance levels.

6.3 Results

Post-harvest lettuce samples from a commercial organic Maryland farm, harvested by farm personnel, were treated with different washing methods, including unwashed, washed with sanitizers (by farm personnel following farm procedures), bleach, and tap water. Indicator bacterial counts were measured at both Day 1 and Day 6 after harvest. Five bacteria indicators: total coliforms, *E. coli*, APC, yeast and mold were tested. No total coliforms and *E. coli* were detected in any of the samples. Consistent with a previous study [99], we found that although washing significantly reduced bacterial load initially (APC, yeast, and molds; $p<0.05$), the difference between unwashed and washed (with sanitizer or tap water) samples become much smaller after 5 days of storage in all cases (Fig. 6.1). For example, there were significant differences between unwashed and washed samples of all three bacterial indicators bacteria at day 1, yet only mold shows significant difference at day 6.

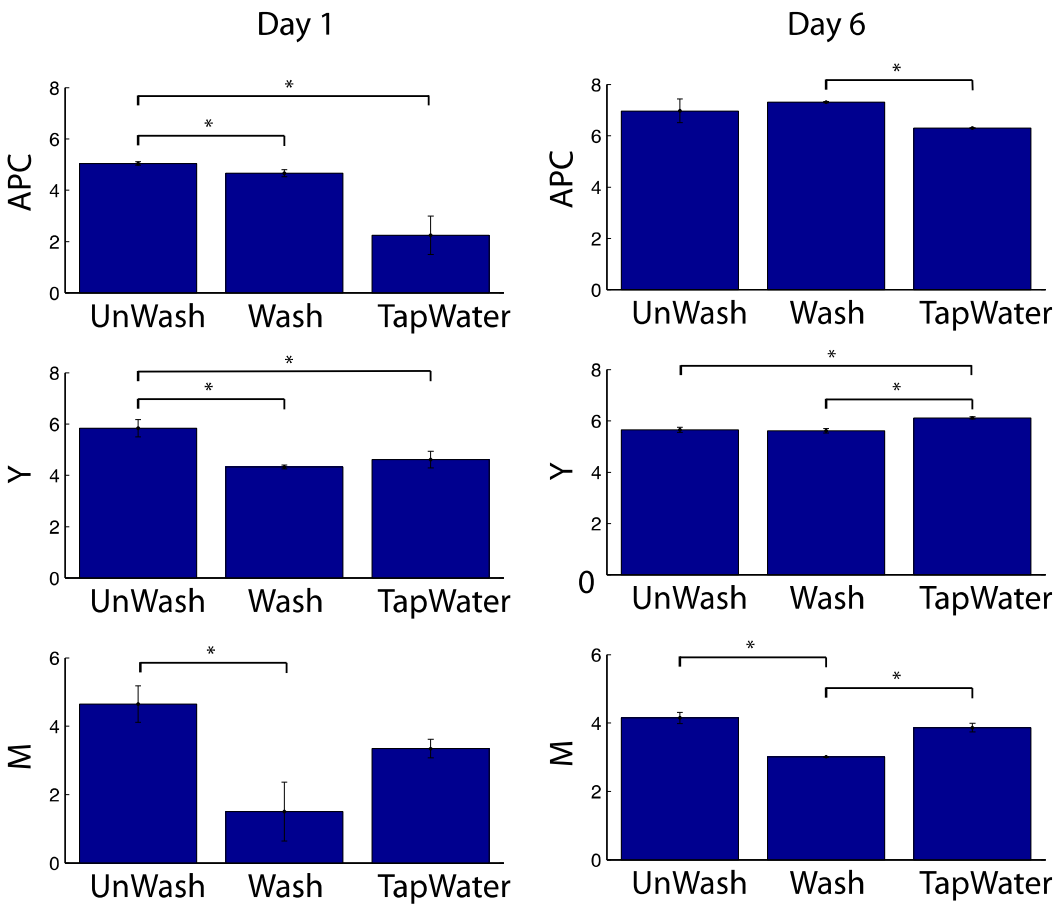


Figure 6.1 Counts of indicator bacteria on samples using different washing treatments (unwashed, washed with sanitizer, washed with tap water) before (left) and after (right) 5 days of storage. * $p<0.05$.

To investigate whether the epiphytic bacterial community is also altered by different post-harvest washing procedures, ARISA was performed to generate bacterial community profiles. Examples of electropherograms are shown in Fig. 6.2, where fluorescence of amplified DNA of the intergenic spacer region (ISR) between 16S and 23S subunits of the rRNA genes was plotted against the size. The community profile was represented as either presence-absence data or relative abundance (relative fluorescence) data from distinct ISR sizes between 80 bp and 600 bp.

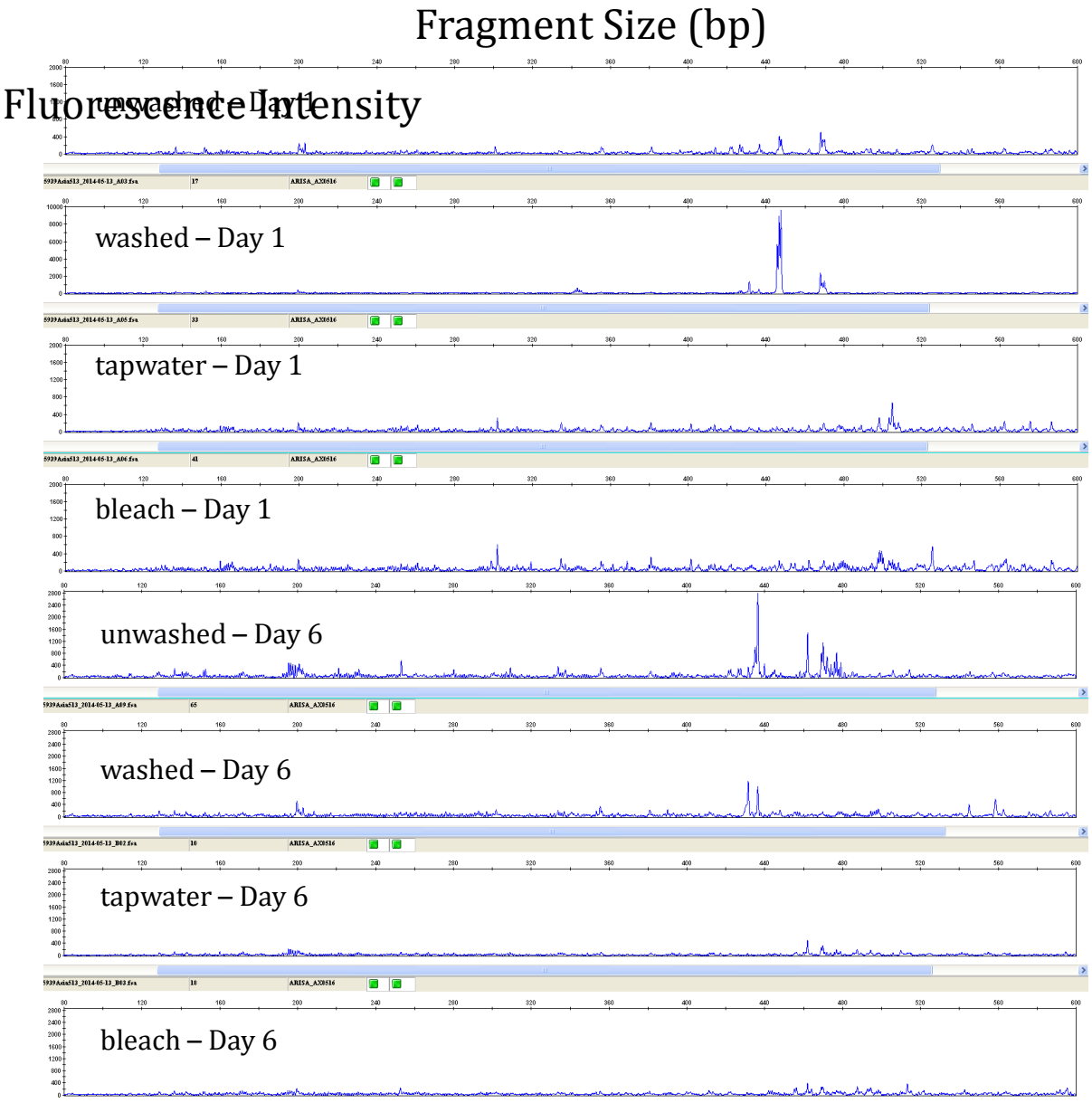


Figure 6.2 Example electropherograms of bacterial community profile from experiment 1. The top four panels are unwashed, washed with sanitizer, tap water and bleach samples from day 1, and the bottom four panels are the same treatments at post-harvest day 6.

The relationships among epiphytic bacterial communities of different washing conditions were assessed by MDS ordination. The MDS map using both relative abundance data (Fig. 6.3A) and presence-absence data (Fig. 6.3B) revealed a clear separation between

samples collected from the first and second experiments, especially for the unwashed samples at post-harvest day 1. Using pairwise test, the difference of Unwashed-Day1 samples between the two experiments was highly significant both using the presence-absence data (R= 0.979, =0.018) and abundance data (R= 0.908, p=0.018). A list of significantly different treatments pairs are shown in the Appendix III. Since the goal of this study was to evaluate the impact of different post-harvest processing methods, we chose not to focus on the difference across experiments, which represents temporal changes in lettuce phyllosphere communities, but rather separately discussed the first and second experiments to look for patterns due to post-harvest treatments and storage.

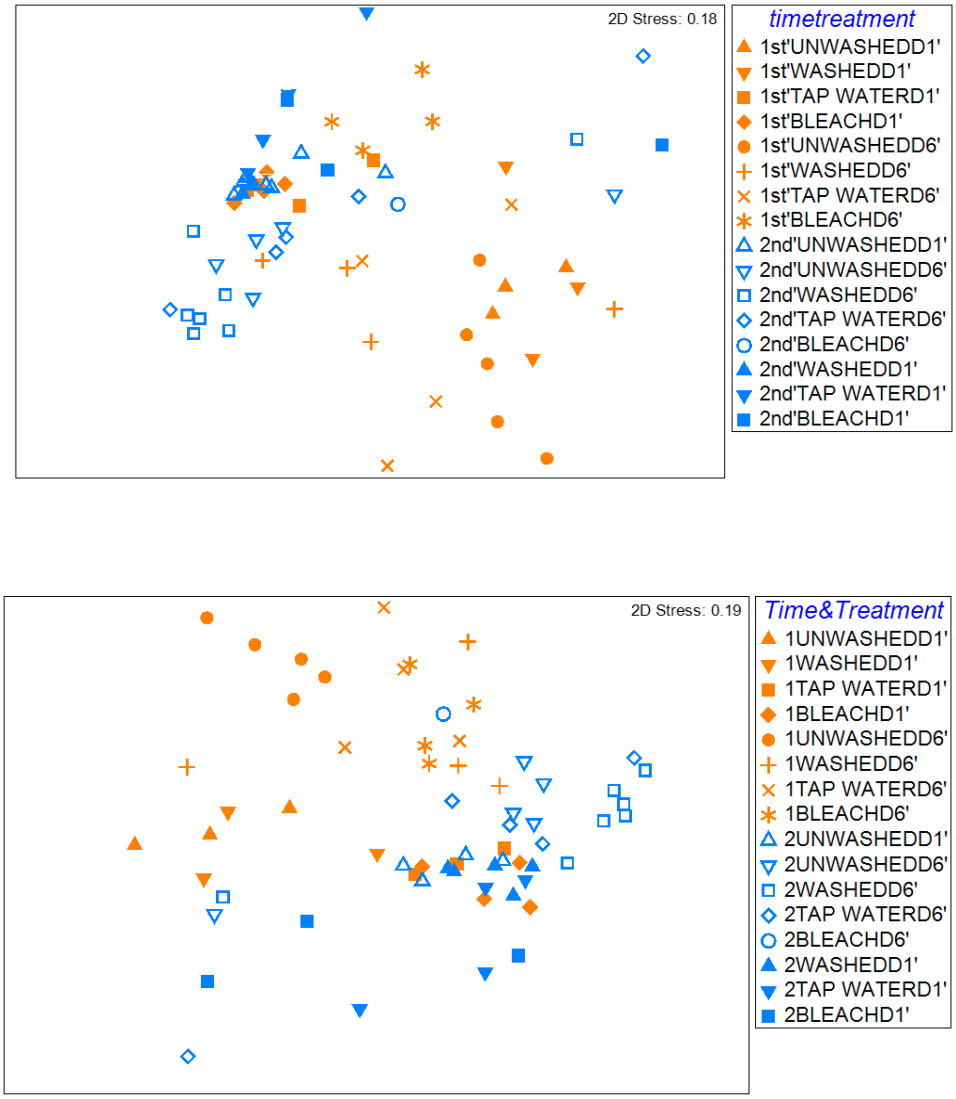


Figure 6.3 Non-metric MDS plot of (A) relative abundance and (B) presence-absence data from ARISA profiles of bacterial communities under different washing conditions and storage durations.

We next compared different post-harvest treatments (unwashed, washed, tap water and bleach) on bacterial community structure both right after harvest (Day 1) and after storage of 5 days (Day 6). For both experiments, there was a significant difference among treatments using relative abundance data (global R=0.70, p<0.001, experiment 1; global

R=0.30, p<0.001, experiment 2 Fig. 6.4 (C-D)). The difference was apparent in presence absence data also in experiment 2, but less prominent in experiment 1 (global R=0.084, p=0.13, experiment 1; global R=0.28, p<0.005 experiment 2 Fig. 6.4 (A-B)). A non-significant p-value was obtained in experiment 1, which implies higher similarity among bacterial OTU composition from different treatments, but more pronounced differences in community structure. In experiment 2, both bacterial community and structure were impacted. The MDS plots show both separations due to treatment and storage times (Fig. 6.4 (C and D)).

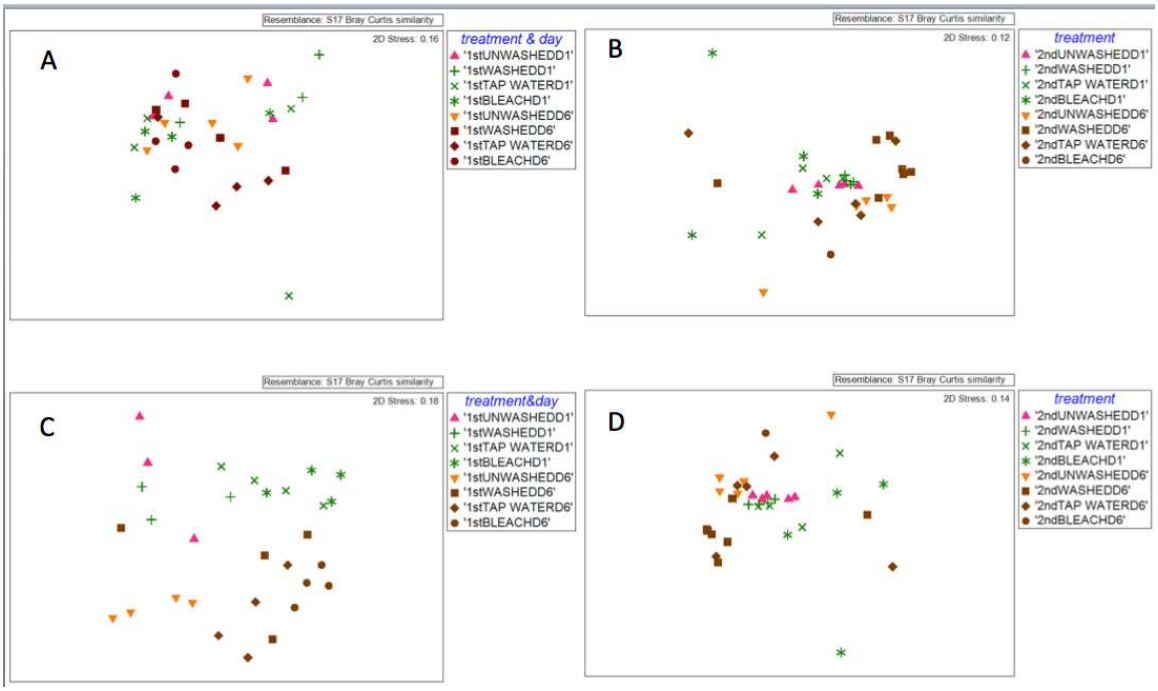


Figure 6.4 Impact of different post-harvest treatments on bacterial communities. Non-metric MDS plots of presence-absence (A-B) data and relative abundance data (C-D) from ARISA profiles of bacterial communities with different post-harvest treatments. (A) and (C) are using data from experiment 1. (B) and (D) are using data from experiment 2.

One aim of the study was to investigate whether the difference among treatment only occurred at Day 1 or represented a persistent, long-lasting alteration on bacterial community structure. The dissimilarity among treatments at Day 1 and Day 6 were separately evaluated. The differences among treatments were still significant after 5 days of storage. The global R value changed only slightly from post-harvest day 1 (Global R = 0.62, $p<0.001$, experiment 1; Global R = 0.18, $p<0.05$, experiment 2) to post-harvest day 6 (Global R = 0.56, $p<0.001$, experiment 1; Global R = 0.22, $p<0.05$, experiment 2). R statistic for pairs could be checked in Appendix III.

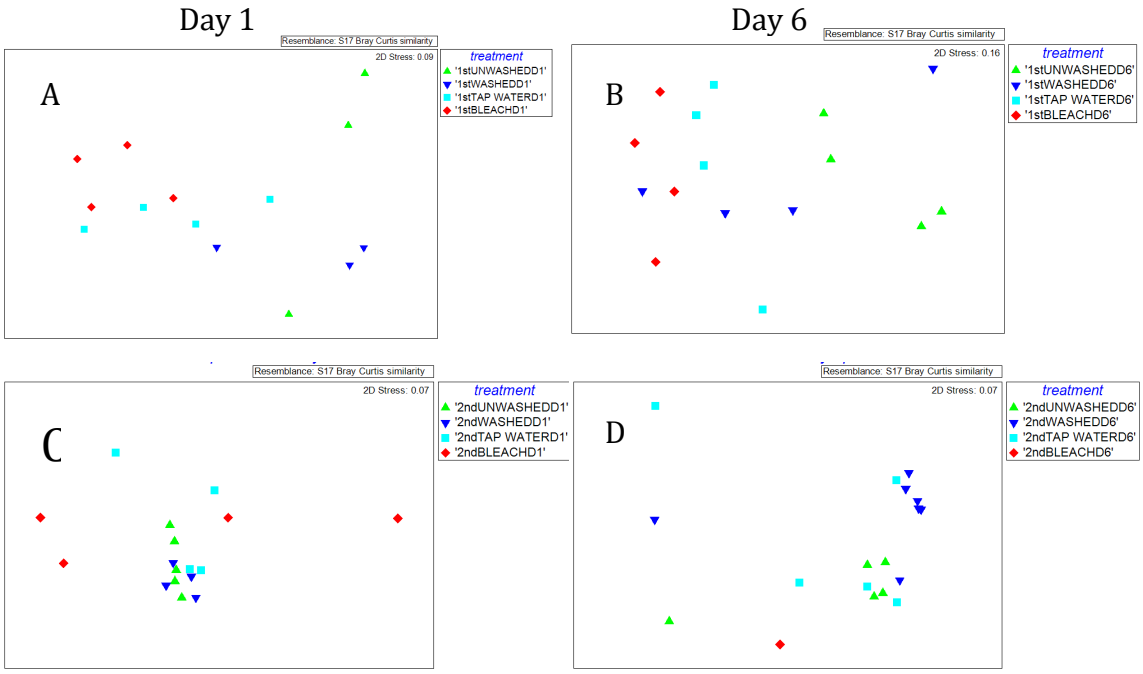


Figure 6.5 Impact on community structure by different washing treatments. The non-metric MDS plot is separately generated for Day 1 (left) and Day 6 (right) using relative abundance data. The top row is experiment 1 (A-B), and the second row is experiment 2 (C-D). For both experiments, the differences among treatments are significant even after 5 days of storage.

6.4 Discussion

A diverse set of microorganisms colonize the surface of fresh fruit and vegetables [72], and each produce type has its distinct bacterial community profile [77]. Nevertheless, most of the studies in food safety have focused on a relatively small set of pathogenic bacteria. A number of studies showed that interactions with epiphytic bacteria can either facilitate or suppress the growth of pathogens [79,84,85]. It is important to evaluate whether different post-harvest treatments will alter the bacterial community profile.

Our results demonstrated that the impact of different washing methods on the epiphytic bacterial community persists even after 5 days of storage (Fig. 6.5). In contrast, the reductions in indicator bacteria counts for samples under different treatments generally diminished after storage and sometimes increased (Fig. 6.1). Therefore, the indicator bacterial counts do not reflect the true effect of post-harvest rinsing treatments and storage. This is particularly problematic since indicators such as *E. coli* are used to assess food safety risk, even though they often poorly correlate with actual pathogens [137]. To completely assess the impact of a post-harvest treatments on the phyllosphere biome, we need not only to monitor a small set of indicator bacteria, but also evaluate the diversity of a microbial community. Methods such as community fingerprinting, provide a quick and inexpensive method to describe community profiles of produce samples for food safety assessment.

We observed significant variation in bacterial community fingerprints across experiments even for unwashed samples (before any post-harvest treatment). There is a clear separation between samples collected from the first and second experiment. This separation represents variation of initial epiphytic bacterial communities across experiments, which may be due to the temporal difference of environmental conditions (e.g. temperature, precipitation) or growing conditions. This finding does not necessarily contradict recent studies that suggest each produce type has distinct bacterial community profile [77], because we do not know whether this variation across experiment is larger than the variation across produce types.

Despite the initial difference of unwashed samples across experiments, we observed that washing significantly changed the community structure both initially and after 5 days of storage. This alteration is better revealed using relative abundance data than presence-absence data. The discrepancy between presence-absence data and relative-abundance data suggests that the presence-absence of bacterial species is less affected by different treatments than the relative abundance (structure of the community) of different species within the community, at least in experiment 1. Therefore, the species that were suppressed initially may resurge under appropriate conditions.

It still remains to be explored how changes in epiphytic bacterial community structure as a result of different treatments might affect contamination risk of pathogenic bacteria, or susceptibility to contamination. To achieve this, it is necessary to identify individual species of bacteria, and use existing knowledge about interaction between pathogen and other bacteria to evaluate whether the survival and growth of pathogenic bacteria will be

altered. Since ARISA is a community fingerprinting technique that does not result in identification of individual microbial species, other molecular biology techniques, such as DNA sequencing, would be needed to identify individual epiphytic bacterial species.

Chapter 7 Summary, Conclusions, and Future Directions

7.1 General Findings of Study

Microbiological safety of produce from small organic farms in the Mid-Atlantic region was systematically assessed in this study. Leafy green samples were found to have the highest bacterial counts among different types of produces. At the pre-harvest stage, different types of mulching were identified to have a significant effect on the survival of indicator bacteria. At the post-harvest stage, the commonly used washing procedure failed to suppress the survival of indicator bacteria. In contrast, washing can lead to significant increases in bacterial counts if sanitizer is not used properly. Further analysis with community fingerprinting techniques revealed that washing has a long-lasting impact on the epiphytic bacterial community structure, which was not captured by counts of indicator bacteria.

7.2 Conclusions

- Washing without sanitizer increased the indicator bacterial counts on leafy green samples
- Mulching is identified as an important pre-harvest factor for the survival of indicator bacteria. Different types of mulching has significantly different effects on the bacterial counts both on the leaf samples and in the soil samples

- Post-harvest washing can significantly alter the epiphytic bacterial community. Unlike indicator bacterial counts, the effect persisted after 5 days of storage

7.3 Future Directions

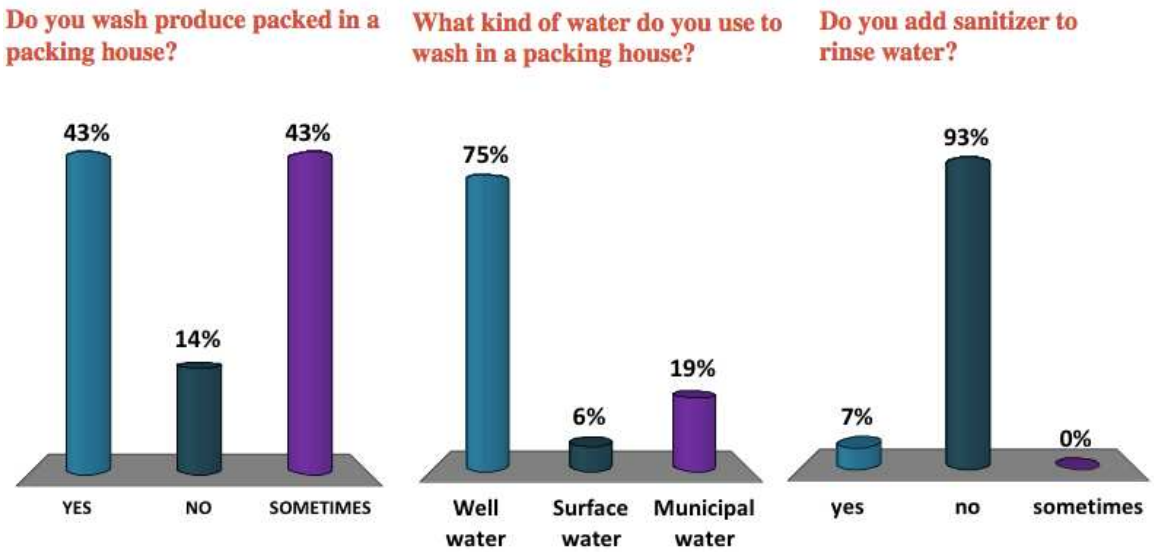
Many gaps still exist in investigating the impact of different pre- and post-harvest practices on the risk of contamination by pathogenic bacteria. Several future directions have been identified and listed below.

Among the growing practices, mulching is important fact. But only relying on the bacteria indicators are not enough, to understand better, the mulch effects on the bacteria community on lettuce and soil need to be explored. Further, more commonly used mulch types for produce growing need to be assessed, such as straw and hay. In addition to lettuce, other produce types are waiting to be assessed as well.

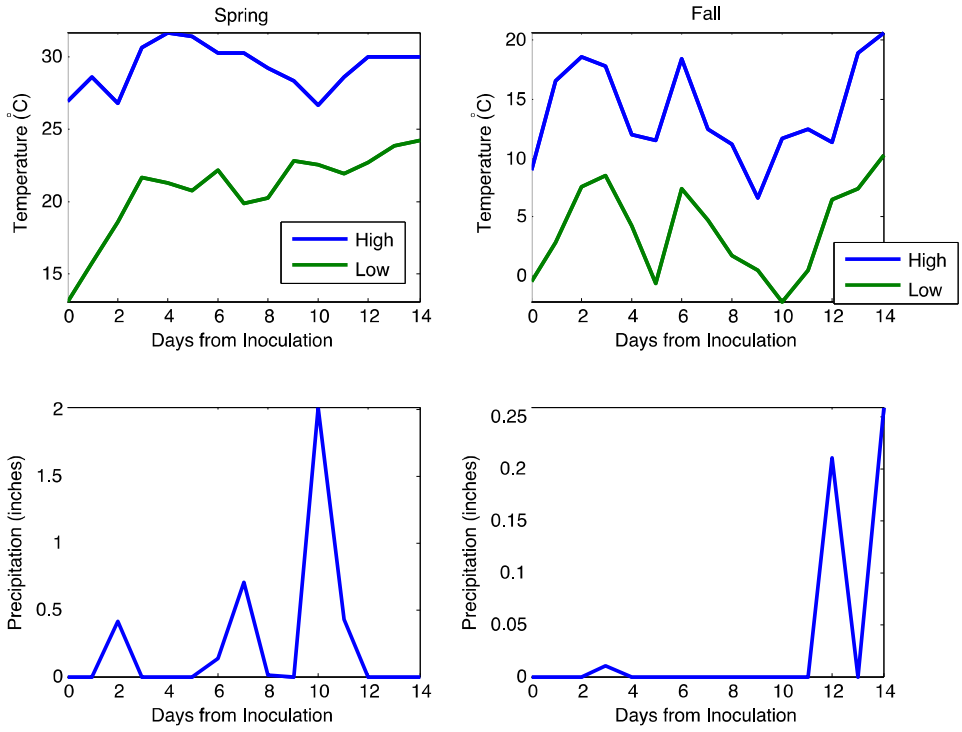
Among handling practices, washing methods differ a lot. And it is a final step to reduce food safety risks before eating for some produce, such as spring mix salad. More work need to be done to better understand the bacteria community for the microbial safety. To achieve this, it is necessary to identify not only the individual species of bacteria and also interaction between pathogen and other bacteria to evaluate whether the survival and growth of pathogenic bacteria will be altered. Furthermore, the findings in the current study based on the ARISA results should be verified by other molecular biology techniques, such as DNA sequencing.

Appendices

Appendix I Survey of Organic Farm Management Practice in Maryland



Appendix II Temperature and Precipitation Data



Appendix II: Temperature (top) and precipitation (bottom) during the experiment for both the spring and fall season. Weather data was extracted from the WyeREC database online.

Appendix III Pairwise Test on ARISA Profiles
Presence-Absence data, experiment 1

Pairwise Tests		
	R	Significance
Groups	Statistic	Level %
'1stUNWASHEDD1', '1stWASHEDD1'	0.037	37.1
'1stUNWASHEDD1', '1stTAP WATERD1'	-0.073	57.1
'1stUNWASHEDD1', '1stBLEACHD1'	-0.047	48.6
'1stUNWASHEDD1', '1stUNWASHEDD6'	-0.059	52.4
'1stUNWASHEDD1', '1stWASHEDD6'	0.094	28.6
'1stUNWASHEDD1', '1stTAP WATERD6'	0.521	5.7
'1stUNWASHEDD1', '1stBLEACHD6'	0.198	22.9
'1stWASHEDD1', '1stTAP WATERD1'	-0.028	45.7
'1stWASHEDD1', '1stBLEACHD1'	0.157	20
'1stWASHEDD1', '1stUNWASHEDD6'	0.082	28.6
'1stWASHEDD1', '1stWASHEDD6'	0.315	14.3
'1stWASHEDD1', '1stTAP WATERD6'	0.611	5.7
'1stWASHEDD1', '1stBLEACHD6'	0.463	5.7
'1stTAP WATERD1', '1stBLEACHD1'	-0.125	74.3
'1stTAP WATERD1', '1stUNWASHEDD6'	-0.072	69
'1stTAP WATERD1', '1stWASHEDD6'	0.031	34.3
'1stTAP WATERD1', '1stTAP WATERD6'	0.031	34.3
'1stTAP WATERD1', '1stBLEACHD6'	0	48.6
'1stBLEACHD1', '1stUNWASHEDD6'	-0.113	70.6
'1stBLEACHD1', '1stWASHEDD6'	0.078	25.7
'1stBLEACHD1', '1stTAP WATERD6'	0.172	20
'1stBLEACHD1', '1stBLEACHD6'	-0.073	77.1
'1stUNWASHEDD6', '1stWASHEDD6'	0.028	37.3
'1stUNWASHEDD6', '1stTAP WATERD6'	0.406	4
'1stUNWASHEDD6', '1stBLEACHD6'	-0.128	80.2
'1stWASHEDD6', '1stTAP WATERD6'	0.167	22.9
'1stWASHEDD6', '1stBLEACHD6'	0.302	11.4
'1stTAP WATERD6', '1stBLEACHD6'	0.542	5.7

Relative abundance data, experiment 1

Pairwise Tests		
	R	Significance
Groups	Statistic	Level %
'1stUNWASHEDD1', '1stWASHEDD1'	0.593	10
'1stUNWASHEDD1', '1stTAP WATERD1'	0.796	2.9
'1stUNWASHEDD1', '1stBLEACHD1'	0.926	2.9
'1stUNWASHEDD1', '1stUNWASHEDD6'	0.815	2.9
'1stUNWASHEDD1', '1stWASHEDD6'	0.537	8.6
'1stUNWASHEDD1', '1stTAP WATERD6'	0.722	2.9
'1stUNWASHEDD1', '1stBLEACHD6'	0.889	2.9
'1stWASHEDD1', '1stTAP WATERD1'	0.389	8.6
'1stWASHEDD1', '1stBLEACHD1'	0.944	2.9
'1stWASHEDD1', '1stUNWASHEDD6'	1	2.9
'1stWASHEDD1', '1stWASHEDD6'	0.315	11.4
'1stWASHEDD1', '1stTAP WATERD6'	0.889	2.9
'1stWASHEDD1', '1stBLEACHD6'	1	2.9
'1stTAP WATERD1', '1stBLEACHD1'	0.115	17.1
'1stTAP WATERD1', '1stUNWASHEDD6'	1	2.9
'1stTAP WATERD1', '1stWASHEDD6'	0.354	8.6
'1stTAP WATERD1', '1stTAP WATERD6'	0.771	2.9
'1stTAP WATERD1', '1stBLEACHD6'	0.854	2.9
'1stBLEACHD1', '1stUNWASHEDD6'	1	2.9
'1stBLEACHD1', '1stWASHEDD6'	0.552	2.9
'1stBLEACHD1', '1stTAP WATERD6'	0.844	2.9
'1stBLEACHD1', '1stBLEACHD6'	0.906	2.9
'1stUNWASHEDD6', '1stWASHEDD6'	0.635	2.9
'1stUNWASHEDD6', '1stTAP WATERD6'	0.708	2.9
'1stUNWASHEDD6', '1stBLEACHD6'	0.979	2.9
'1stWASHEDD6', '1stTAP WATERD6'	0.156	14.3
'1stWASHEDD6', '1stBLEACHD6'	0.458	2.9
'1stTAP WATERD6', '1stBLEACHD6'	0.25	11.4

Presence-Absence data, experiment 2

Pairwise Tests		
	R	Significance
Groups	Statistic	Level %
'2ndUNWASHEDD1', '2ndUNWASHEDD6'	0.37	0.8
'2ndUNWASHEDD1', '2ndWASHEDD6'	0.498	0.6
'2ndUNWASHEDD1', '2ndTAP WATERD6'	0.26	1.6
'2ndUNWASHEDD1', '2ndBLEACHD6'	0.92	16.7
'2ndUNWASHEDD1', '2ndWASHEDD1'	0.006	36.5
'2ndUNWASHEDD1', '2ndTAP WATERD1'	0.113	17.5
'2ndUNWASHEDD1', '2ndBLEACHD1'	0.344	1.6
'2ndUNWASHEDD6', '2ndWASHEDD6'	0.224	6.2
'2ndUNWASHEDD6', '2ndTAP WATERD6'	0.08	18.3
'2ndUNWASHEDD6', '2ndBLEACHD6'	0.2	33.3
'2ndUNWASHEDD6', '2ndWASHEDD1'	0.431	2.4
'2ndUNWASHEDD6', '2ndTAP WATERD1'	0.475	1.6
'2ndUNWASHEDD6', '2ndBLEACHD1'	0.384	2.4
'2ndWASHEDD6', '2ndTAP WATERD6'	0.147	13.4
'2ndWASHEDD6', '2ndBLEACHD6'	0.429	25
'2ndWASHEDD6', '2ndWASHEDD1'	0.422	1.2
'2ndWASHEDD6', '2ndTAP WATERD1'	0.485	1.2
'2ndWASHEDD6', '2ndBLEACHD1'	0.485	1.5
'2ndTAP WATERD6', '2ndBLEACHD6'	0.1	50
'2ndTAP WATERD6', '2ndWASHEDD1'	0.25	5.6
'2ndTAP WATERD6', '2ndTAP WATERD1'	0.2	7.9
'2ndTAP WATERD6', '2ndBLEACHD1'	0.047	30.2
'2ndBLEACHD6', '2ndWASHEDD1'	1	20
'2ndBLEACHD6', '2ndTAP WATERD1'	0.708	20
'2ndBLEACHD6', '2ndBLEACHD1'	-0.042	60
'2ndWASHEDD1', '2ndTAP WATERD1'	0.333	2.9
'2ndWASHEDD1', '2ndBLEACHD1'	0.417	2.9
'2ndTAP WATERD1', '2ndBLEACHD1'	-0.021	54.3

Relative abundance data, experiment 2

Pairwise Tests		
	R	Significance
Groups	Statistic	Level %
'2ndUNWASHEDD1', '2ndUNWASHEDD6'	0.408	0.8
'2ndUNWASHEDD1', '2ndWASHEDD6'	0.526	0.6
'2ndUNWASHEDD1', '2ndTAP WATERD6'	0.348	0.8
'2ndUNWASHEDD1', '2ndBLEACHD6'	1	16.7
'2ndUNWASHEDD1', '2ndWASHEDD1'	0.081	27.8
'2ndUNWASHEDD1', '2ndTAP WATERD1'	0.169	12.7
'2ndUNWASHEDD1', '2ndBLEACHD1'	0.45	0.8
'2ndUNWASHEDD6', '2ndWASHEDD6'	0.327	2
'2ndUNWASHEDD6', '2ndTAP WATERD6'	0.088	11.9
'2ndUNWASHEDD6', '2ndBLEACHD6'	0.2	33.3
'2ndUNWASHEDD6', '2ndWASHEDD1'	0.394	3.2
'2ndUNWASHEDD6', '2ndTAP WATERD1'	0.4	1.6
'2ndUNWASHEDD6', '2ndBLEACHD1'	0.322	4.8
'2ndWASHEDD6', '2ndTAP WATERD6'	0.182	10.1
'2ndWASHEDD6', '2ndBLEACHD6'	0.429	25
'2ndWASHEDD6', '2ndWASHEDD1'	0.437	1.5
'2ndWASHEDD6', '2ndTAP WATERD1'	0.45	2.1
'2ndWASHEDD6', '2ndBLEACHD1'	0.501	1.2
'2ndTAP WATERD6', '2ndBLEACHD6'	0.08	50
'2ndTAP WATERD6', '2ndWASHEDD1'	0.206	7.1
'2ndTAP WATERD6', '2ndTAP WATERD1'	0.119	18.3
'2ndTAP WATERD6', '2ndBLEACHD1'	0.125	24.6
'2ndBLEACHD6', '2ndWASHEDD1'	1	20
'2ndBLEACHD6', '2ndTAP WATERD1'	0.333	40
'2ndBLEACHD6', '2ndBLEACHD1'	-0.042	60
'2ndWASHEDD1', '2ndTAP WATERD1'	0.125	25.7
'2ndWASHEDD1', '2ndBLEACHD1'	0.354	2.9
'2ndTAP WATERD1', '2ndBLEACHD1'	0.109	28.6

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