Colonisation of lettuce by *Listeria monocytogenes* and its biofilm formation

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Abstract

Foodborne outbreaks associated with fresh produce due to pathogenic bacteria such as *Listeria monocytogenes* (*L. monocytogenes*) are increasing. A survey conducted as part of this study showed 5% of bagged lettuces sold at retail outlets contained *L. monocytogenes*. The ability of *L. monocytogenes* to form biofilms is a concern in terms of fresh produce safety. The use of hydroponics (growing plants in nutrient solution without using soil) in fresh produce production is a more controlled environment than soil, and therefore may be easier to prevent pathogen contamination. The aim of this study was to determine whether hydroponic grown lettuce and UV-C stress can reduce the colonisation, growth and biofilm formation of three fresh produce related strains of *L. monocytogenes*: PFR O8A06 (coleslaw isolate), PFR O8A07 and PFR O8A08 (cabbage isolates) on lettuce.

Evaluation of the nutrient content and indigenous microbiota between hydroponic and soil grown lettuces revealed significant differences. 16S rRNA amplicon sequencing of the bacterial community associated with hydroponically grown lettuce showed that it was more diverse than soil-grown lettuce. *Pseudomonas* was found to be the dominant bacteria on hydroponically grown lettuce while *Bacillus* was found to be dominant in soil grown lettuce.

There was no significant difference ($p > 0.05$) in *L. monocytogenes* attachment to both hydroponic and soil grown lettuce leaves under minimal exposure times. Exposure of lettuce to 5 log CFU/ml for just one second resulted in at least 0.77 log CFU/cm^2 attachment. *L. monocytogenes* was able to survive and grow on both lettuce leaf surfaces at 4 and 10°C. Both hydroponic and soil grown lettuce leaf extracts enhanced the survival, growth and biofilm

formation of *L. monocytogenes* on stainless steel coupons, representing surfaces in lettuce processing plants. The results of this study demonstrate the ability of *L. monocytogenes* to colonize and form biofilms on lettuce irrespective of the growth system used. Finally, UV-C (1.3 kJm^{-2}) on lettuce produced a stress response in the plants that reduced *L. monocytogenes* attachment, survival and growth at pre-harvest. Further exploration of this technique may enhance the microbial safety of lettuce.

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Declaration

The presented thesis is comprised of eight chapters. Partial results of chapters 2, 3, 4, 5, 6 and 7 are structured as manuscripts that have either been submitted or published. Some sections in the materials and methods have been repeated in some chapters, however, the results and discussion are different for each chapter.

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Published Manuscripts

1. **Kyere, E.O.,** Palmer, J., Wargent, JJ., Fletcher, GC., & Flint, S. (2018). Colonisation of lettuce by *Listeria monocytogenes***.** *International Journal of Food Science and Technology*, 54, 14-24.

2. **Kyere, E.O**., Goh, W.Q., Siti, NMZ., Palmer, J., Wargent, J.J., Fletcher, GC., & Flint, S. (2020). A comparison of *Listeria monocytogenes* contamination in bagged and un-bagged lettuce in supermarkets. *LWT-Food Science and Technology*, 134, 110022.

3. **Kyere, E.O.,** Foong, G., Palmer, J., Wargent, JJ., Fletcher, GC., & Flint, S. (2019). Rapid attachment of *Listeria monocytogenes* to hydroponic and soil grown lettuce leaves. *Food Control*, 101, 77-80.

4. **Kyere, E.O.,** Foong, G., Palmer, J., Wargent, JJ., Fletcher, GC., & Flint, S. (2020). Biofilm formation of *Listeria monocytogenes* in hydroponic and soil grown lettuce leaf extracts on stainless steel coupons. *LWT-Food Science and Technology*, 126, 109114.

Submitted Manuscripts

1. **Kyere, E.O.,** Gagic, D., Palmer, J., Wargent, JJ., Fletcher, GC., & Flint, S. (2020). 16S rRNA amplicon sequencing reveals differences in microbiome community composition between soil and hydroponically grown lettuce leaves. *Journal of Applied Microbiology*.

2. **Kyere, E.O.,** Popovich, D.G., Palmer, J., Wargent, JJ., Fletcher, GC., & Flint, S. (2020). Reduction of the attachment, survival and growth of *L. monocytogenes* on lettuce leaves by UV-C stress. *Food Research International*.

List of Conference Presentations

1. Emmanuel Kyere. Reduction of the attachment, survival and growth of *L. monocytogenes* on lettuce leaves by UV-C stress. New Zealand Food Safety Science and Research Centre Spotlight Series. Oral presentation via zoom (Sept 2020).

2. Emmanuel Kyere. Colonisation of lettuce by *Listeria monocytogenes* and its biofilm formation. Massey University Postgraduate Food Science Symposium. Oral presentation (Sept 2020).

3. Emmanuel Kyere. Biofilm formation of *Listeria monocytogenes* in lettuce extracts. New Zealand Microbiological Society (NZMS) Conference, Christchurch, NZ. Poster (Nov 2018).

4. Emmanuel Kyere. Colonisation of lettuce by *Listeria monocytogenes*. NZMS Conference, Auckland, NZ, Poster (Nov 2017).

5. Emmanuel Kyere. Rapid Attachment of *Listeria monocytogenes* to lettuce leaves. Plant Science Central Conference, Palmerston North, New Zealand. Oral (July 2017).

CHAPTER 1 General Introduction

1.1 Rationale and importance

L. monocytogenes is a Gram-positive bacterium and the causative agent of listeriosis with a high mortality rate (Sleator et al., 2009). Listeriosis mostly affects immunocompromised individuals such as the elderly and pregnant women. The symptoms of listeriosis include septicaemia, meningitis and miscarriage in pregnant women (McLauchlin, 1997). The ability of *L. monocytogenes* to grow in a wide temperature range including refrigeration temperatures, survive and grow in a wide pH range and its salt tolerance makes it capable of growing in many different environments (NicAogain & O'Byrne, 2016).

There have been many recalls of ready-to-eat (RTE) vegetables due to *L. monocytogenes* in recent years. There has been at least one recall of leafy green salads due to *L. monocytogenes* annually in New Zealand since 2016 (MPI, 2020). In 4 consecutive years; 2016, 2017, 2018 and 2019, there have been many major vegetable related recalls due to *L. monocytogenes* in the Unites States of America (U.S.A). There were 17 recalls in 2016, 12 recalls in 2017, 31 recalls in 2018 and 7 recalls in 2019 (FDA, 2019). With the advancement of technologies in food safety and novel food control methods, one would expect the number of fresh produce related foodborne outbreaks and recalls reducing but this is not the case.

This rise in leafy green-related food poisoning outbreaks provokes researchers and other stakeholders to conduct more studies about the behaviour of pathogenic bacteria on fresh produce. It is evident that there are many gaps in the knowledge and understanding of how pathogens like *L. monocytogenes* colonise the surface of leafy greens such as lettuce (Kyere et

al., 2019). As a result, the current study addresses some of the gaps in order to enable us to better understand how *L. monocytogenes* colonise lettuce surfaces.

An important concern for fresh produce safety is how produce is processed before consumption. Unlike other food products such as milk and meat, fresh produce is often consumed raw without any heat treatment and therefore there is a higher likelihood for pathogens to thrive on produce surfaces (Iwu & Okoh, 2019). The most common sanitizing treatment given to lettuce and other fresh produce during processing includes washing with water mixed with chlorinated compounds such as sodium hypochlorite, acetic acid, peroxyacetic acid and their derivatives (Bhilwadikar et al., 2019; Neto et al., 2012; Baert et al., 2009). However, several researchers (Murray et al., 2017; Warriner & Namvar, 2013a; Ijabadeniyi et al., 2011) have reported the inefficiency of these washing methods. Also, it can be a common practice for fresh produce processing companies to use higher concentrations of chlorinated compounds in produce washing to attempt to enhance effective sanitization (Gadelha et al., 2019). These concentrations are more than the limit that has been set by regulatory agencies and this practice has been associated with negative effects on the health of consumers (Gadelha et al., 2019).

The fresh produce processing chain from farm to fork is a long chain which comprises pre-harvest, harvest, post-harvest, retail, storage, distribution, transport, marketing and finally consumption (Porat et al., 2018). This long chain provides opportunity for pathogen contamination at several stages from farm to consumer.

The main purpose of this work was to investigate factors that can influence (positively or negatively) the contamination of lettuce with *L. monocytogenes*. Firstly, the prevalence of *L. monocytogenes* in lettuces sold in supermarkets in Palmerston North, New Zealand (NZ) was investigated for the first time in this study. The study compared *L. monocytogenes*

contamination in bagged and un-bagged lettuces sold in NZ supermarkets. It was important for such a survey to be carried out due to the frequent recalls of leafy greens associated with *L. monocytogenes* sold in NZ supermarkets (New Zealand Ministry for Primary Industries, 2020).

Secondly, a comparison was made between hydroponic and soil grown lettuces. The information available in literature suggests that hydroponic grown lettuces are better than soil grown lettuces as they have been grown in a controlled environment. Fresh produce grown in soil has a greater risk of exposure to contamination from sources such as manure and wildlife (Strawn et al., 2013: Lima et al., 2013; Neto et al., 2012; Jablasone et al., 2005). In view of this, analysis of the differences between hydroponic and soil grown lettuce leaves that can have a potential effect on lettuce colonisation by *L. monocytogenes*. The differences tested were lettuce leaf microbiota indigenous to both lettuce types as well as the nutrient content of both lettuce types. Such properties may influence the contamination of lettuce with unwanted microorganisms (Selma et al., 2012; Klerks et al., 2007). Understanding the effect of these properties may suggest ways of mitigating the colonisation of lettuce with pathogens such as *L. monocytogenes*.

The first step in the relationship between the pathogen and the fresh produce is the ability of the pathogen to attach to the fresh produce. After attachment, *L. monocytogenes* is able to establish itself by successfully colonising the produce through biofilm formation (Patel &Sharma, 2010). The third part of this study compared the attachment of *L. monocytogenes* to both hydroponic and soil grown lettuce leaves as well as the survival and growth of *L. monocytogenes* on both lettuce types. The minimum exposure time for *L. monocytogenes* attachment to lettuce was reported for the first time in this study.

One key stage in the fresh produce supply chain is processing the lettuce once harvested. Some foodborne outbreaks associated with fresh produce due to *L. monocytogenes* have implicated

the processing environment as the source of contamination (McCollum et al., 2013). In the fourth part of the study, the potential for the processing plant surfaces contaminated with the juice from lettuces to support *L. monocytogenes* biofilm was examined for the first time in this study, demonstrating the risk of a contamination source that needs to be managed.

The last part of the study describes, for the first time, the use of pre-harvest UV stress to reduce *L. monocytogenes* on lettuces. This part of the study examined using stress response in lettuce generated through exposure to UV light, to reduce *L. monocytogenes* colonisation. UV stressing of some leafy greens such as broccoli has been found to stimulate the plant to produce antimicrobial substances which can prevent pathogen growth (Gamage et al., 2016).

This PhD project reveals a better understanding of the factors that affect the colonisation of lettuce by *L. monocytogenes* and the effect of UV stress on lettuce. This information and knowledge will be useful to improve the safety of lettuce. The research demonstrates the importance of exploring alternative fresh produce food control methods capable of preventing colonisation by *L. monocytogenes* will be essential to the fresh produce industry.

1.2 Research questions and hypotheses

1.2.1 Questions

-What is the frequency of contamination of lettuce in supermarkets?

-Are there any differences between the microbial composition of hydroponic and soil grown lettuce which can affect colonisation by *L. monocytogenes*?

-Are there any differences between the nutrient content of hydroponic and soil grown lettuce which can affect *L. monocytogenes* colonisation?

-Are there differences in the attachment ability of *L. monocytogenes* to hydroponic and soil grown lettuce and what is the minimum exposure time for *L. monocytogenes* to attach to lettuces?

-What are the conditions which affect the survival and biofilm formation of *L. monocytogenes* on hydroponic grown and soil grown lettuce and lettuce extracts?

-Do lettuce extracts support the biofilm growth of *L. monocytogenes* on stainless steel surfaces?

-How does pre-harvest UV stress on lettuce affect *L. monocytogenes* survival and biofilm formation?

1.2.2 Hypotheses

-Different growth systems used for lettuce cultivation would influence the bacteria community associated with lettuce leaves.

-Different growth systems used for lettuce cultivation would influence the nutrient content in lettuce which can affect colonisation by *L. monocytogenes*.

-*L. monocytogenes* will show different attachment, survival and growth on hydroponic and soil grown lettuce leaves.

-Lettuce leaf extracts can support the biofilm formation of *L. monocytogenes* on produce processing equipment such as stainless steel.

-UV stress can reduce the attachment, survival and growth of *L. monocytogenes* on lettuce.

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CHAPTER 2 Literature Review

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Abstract

Foodborne illnesses involving ready-to-eat (RTE) vegetables are increasing. Lettuce is the third most consumed fresh vegetable in the United States (US) with worth approximately $1.9 billion, making it the most valuable leafy crop. Previous reviews have described the survival of pathogenic bacteria such as *E. coli* O157:H7 and *Salmonella enterica* on different RTE vegetables, but the colonisation of lettuce by *L. monocytogenes* has received limited attention. *L. monocytogenes* has high mortality compared to other foodborne pathogens such as *Salmonella*. This review summarises recent studies on the mechanisms of attachment, biofilm formation and colonisation of lettuce leaves by *L. monocytogenes*. We discuss various factors that affect colonisation of lettuce by *L. monocytogenes* in terms of the number of bacteria that can be recovered after inoculation, the effect of washing, lettuce microbiota, different radiation treatments and cultivation systems on the recovery of *L. monocytogenes*. We propose strategies that can be used to minimise the colonisation of lettuce by *L. monocytogenes* to enhance food safety.

2.1 Introduction

The rise in leafy green-related food poisoning outbreaks has brought about an increased enthusiasm for studying the behaviour of enteric pathogens on fresh produce (Yaron & Romling, 2014). Stakeholders are trying to understand the causes for the increase in leafy green foodborne outbreaks and how they can be controlled. This can be seen in the recent formation and implementation of institutes and legislations such as the Fresh Produce Safety Australia/New Zealand and the United States, Food and Drug Administration Food Safety Modernisation Act (FSMA) Final Rule on Produce Safety (USA). Changing standards of hygiene, consumers perception, trends towards more fresh produce and growing immunocompromised sectors of the population are likely factors contributing to the increasing incidence of food safety concerns with fresh produce (Yu et al., 2018; Kearney, 2010).

Listeria monocytogenes is a Gram-positive bacterium that has been involved in foodborne outbreaks and recalls involving lettuce. *L. monocytogenes* is a ubiquitous bacterium which can be found in different environments such as soil, plants, water and food processing environments. The ability of *L. monocytogenes* to thrive in a wide range of temperatures and to survive with or without oxygen aids its survival and ability to spread to other sources (NicAogain & O'Byrne, 2016). One important current concern with the contamination of fresh produce by *L. monocytogenes* is the frequency at which they are been identified in RTE vegetables sold at retail.

Recent studies (Xylia et al., 2019; Burnett et al., 2020) about the prevalence of *L. monocytogenes* in fresh produce sold at retail as well as retail produce environments have been conducted. The prevalence of *L. monocytogenes* in ready-to-eat (RTE) salads sold in supermarkets in Cyprus was found to be 3.70%. The prevalence of *L. monocytogenes* in environmental samples found in retail was 4.4% (Burnett et al., 2020). *L. monocytogenes* was

found on both contact and non-food contact surfaces in retail stores in the US. Genetically similar strains were reported to be found in multiple stores (Burnett et al., 2020). These results suggest the possibility of cross contamination in retail stores and call for the need for effective control measures to be followed in the fresh produce industry as well as fresh produce sold in supermarkets.

Lettuce contaminated with *L. monocytogenes* caused 19 people to get sick and left one person dead in an outbreak that occurred in 2015-2016, in nine different states across the US. The source was linked to a processing facility in Springfield, Ohio (Centers for Disease Control (CDC, 2016).

There have been several recent recalls associated with fresh produce due to *L. monocytogenes* in New Zealand (NZ) (MPI, 2020). Speirs Food (a NZ food company) recalled specific RTE salad products due to *L. monocytogenes*. Coleslaw produced by Pams (a NZ food company) was also recalled due to *L. monocytogenes*. All the frozen vegetable products from Bell Farms brand were recalled due to *L. monocytogenes* (MPI, 2020). The biggest salad producer (LeaderBrand) in New Zealand recalled all its products in March 2017 due to a positive test for *Listeria* on the lettuce they produced (MPI, 2020). Apart from lettuce, other leafy greens and RTE vegetables have been associated with *L. monocytogenes* outbreaks and recalls (Oliveira et al., 2011). The most deadly foodborne outbreak in the US occurred in 2011 where four strains of *L. monocytogenes* associated with the outbreak were traced back to whole cantaloupes and packing equipment on Jensen Farms in Colorado (CDC, 2012), resulting in 147 people infected with 33 deaths. The most recent *L. monocytogenes* outbreak (March 2018) in Australia was linked to rockmelon. It resulted in five deaths with several others getting infected (Australian Institute of Food Safety, 2018). According to the FDA, there have been more than 40 recalls in

leafy greens, fruits and vegetables products due to *L. monocytogenes* in US in the past 3 years (FDA, 2018) (Table 2.1).

Lettuce (*Lactuca sativa* L.) is a widely consumed vegetable and is the seventh most consumed vegetable in US, mostly grown in California and Arizona (Statista, 2019; Krıstkova et al., 2008). The value of lettuce production in US in 2015 was approximately $1.9 billion, making it the most valuable crop in the US (USDA, 2016). According to Mou (2009), there are six main varieties of lettuce. These are crisphead, butterhead, romaine or cos, leaf or cutting lettuce, stem or stalk lettuce and Latin lettuce. Differences between the above- named lettuces depend on the shape of the leaf, size, texture, head formation and stem type (Mou, 2009).

Table 2.1. Recent recalls of leafy greens, fruits and vegetables products due to *L. monocytogenes* in United States.

Product Description	Location
2019	
Salad Products	Hollywood, FL
Salad Products	Houston, TX
2018	
Vegetables	Groveport, OH
Salad and Bowl Products	Indianapolis, IN
Salad Products	Houston, TX
Vegetables	Burnsville, MN
Vegetables	Corona, CA
Salad Products	Houston, TX
Salads	Sacramento, CA
Salads	Green Cove Springs, FL
Salads	Lewisville, TX
2017	
Red/green apple slices Sliced apples	Detroit, MI
Frozen whole green beans	Virginia
Salad kit	Idaho
Bagged broccoli florets	Warrendale, PA
Deli broccoli salads	Colorado
Packaged produce	Michigan
Soybean sprout	Virginia
Fresh spinach	Louisville, KY
Frozen sweet peas	Manitowoc, WI
2016	
Ready-to-eat salads	New York
Fresh-cut vegetables	Texas
Frozen cut corn	Lancaster, PA
Organic mixed vegetables	Chicago, IL
Cut green beans	East Petersburg, PA
Potato salad	Washington
Frozen mixed vegetables	Retail Stores Nationwide
Frozen green peas	Southern California, CA
Sunflower seeds, pumpkin seeds and almonds	Multistate
Sunflower seeds	Lakeville, MN
Frozen green and baby lima beans and organic mixed vegetables	Washington
Oriental salad with sesame ginger dressing	Phoenix, AZ
Green beans	Corvallis, OR
Frozen yellow cut corn	South Carolina

Source: https://www.fda.gov/Safety/Recalls/MajorProductRecalls/ucm581921.htm

Consumption of leafy green vegetables such as lettuce is popular as they are healthy and available throughout the year (Pollack, 2001). Lettuce has various nutritional benefits. It contains vitamin C, iron, folate and fibre. It lowers cholesterol, and it is believed to prevent diabetes and reduce inflammation (Willcox et al., 2009). Kim et al. (2016a) reported that crisphead or iceberg lettuce has the lowest nutritional value whereas butterhead and romaine lettuce were the highest in terms of their nutritional value among the lettuce types in the US.

Consumption of leafy greens in US between 1973 and 2012 was reported to result in 20003 illness and 1030 hospitalisations, making it the highest compared to illness associated with other food types (Herman et al., 2015). As lettuce is mostly consumed raw without cooking, this exacerbates the food safety risk compared with vegetable products that are cooked. Sources of contamination include humans, animals, manure, compost and irrigation water (Jung et al., 2014). After contamination of lettuce by *L. monocytogenes*, bacteria attach to lettuce leaf surfaces, persist and colonise the lettuce. Several researchers have reported the inadequacy of washing using various chemicals to completely remove pathogens from the surfaces of leaves (Bhilwadikar et al., 2019; Luo et al., 2011; Koseki et al., 2003; Seo & Frank, 1999). Shirron et al., (2009) showed that after washing parsley leaves with sodium dichloroisocyanurate (a slow releasing chlorine product), enteric pathogens can still survive and grow on the surface.

L. monocytogenes can survive and persist on lettuce leaf surfaces in different conditions such as high salt concentration, low oxygen, modified atmosphere etc. (Poimenidou et al., 2016; Beuchat & Brackett, 1990a). Murphy et al. (2016) demonstrated that *L. monocytogenes* thrives on the surface of lettuce leaves grown in peat (a plant growth medium which consists of slow decomposed vegetative matter) supplemented with contaminated food waste. Beuchat & Brackett, (1990a) showed that *L. monocytogenes* grows and multiplies on lettuce stored in a modified atmosphere (3% O_2 and 97% N_2). The ability of many strains of *L. monocytogenes* to

be able to withstand harsh food environments such as high salt concentration, low oxygen and high acidity has been reported (Poimenidou et al., 2016). After *L. monocytogenes* was exposed to lactic acid at a pH of 3.5 for 6 h, significant variability in acid resistance was observed for strains sourced from different environments. *L. monocytogenes* cells on lettuce leaf surfaces for 5 days at 5 °C were able to survive for 360 min after lactic acid treatment but cells that were on the leaves just 24 h became inactivated after 265 min (Poimenidou et al., 2016). This suggests that, under acidic stress, *L. monocytogenes* which colonise lettuce leaves for a longer period are more tolerant to acid stress as compared to colonisers over a shorter time period. This is suggestive of biofilms of bacteria developing resistance to environmental stress. *L. monocytogenes* can translocate to protective sites on the leaf over time to prevent acidic stress (Capozzi et al., 2009). The ability of leaves to exude organic matter over time may also protect attached cells (Brandl et al., 2004).

This review gives a summary of recent studies about the mechanisms of attachment, biofilm formation and colonisation of *L. monocytogenes* strains on lettuce leaves after inoculation, as well as their recovery rate. Due to the limited information on the colonisation of lettuce by *L. monocytogenes*, other leafy greens, as well as other pathogenic bacteria such as Shiga-toxin producing *E. coli* and *Salmonella enterica*, were used as examples. We discuss the various factors that affect colonisation of lettuce by *L. monocytogenes* in terms of the number of bacteria that can be recovered after inoculation, biofilm formation of *L. monocytogenes* in fresh produce, the effect of different washing treatments, effect of leaf surface microbiota, UV radiation and different cultivation systems such as hydroponic systems on the recovery rate. Finally, we propose strategies that can be used to minimise colonisation of lettuce by *L. monocytogenes* to enhance food safety.

2.2 Mechanisms of attachment and colonisation of *L. monocytogenes* strains on lettuce leaves after inoculation

There are various routes where lettuce can be contaminated by *L. monocytogenes*. Possible sources of contamination are people who handle lettuce, retail environment, contaminated irrigation water, packhouse surfaces, rotting vegetable matter, soil, animal or human faeces used as manure and wildlife moving into areas where lettuce is grown (Burnett et al., 2020; Jiang et al., 2004). The first step in fresh produce contamination is bacterial attachment (Cabedo et al., 1997). Attachment is thought to occur in two stages: an initial reversible attachment stage and an irreversible attachment stage (Romantschuk, 1992). Initial attachment is thought to be weak and unspecific. It primarily depends on the interactive forces such as hydrophobic and electrostatic forces between the bacterium and the leaf surface. Initial attachment is also thought to be reversible (Dunne, 2002). A hydrophobic cuticle lines the surfaces of leafy green vegetables which serves as an attraction for hydrophobic bacteria (Patel & Sharma, 2010). Cell surface charge and hydrophobicity of *L. monocytogenes* have been identified as important factors in attachment (Ukuku & Fett, 2002). When *Arabidopsis thaliana* leaves were exposed to a suspension of 10^8 CFU mL^{-1} *L. monocytogenes* for 5 min, 1.52 log CFU cm^{-2} to 2.17 log CFU cm^{-2} of *L. monocytogenes* were recovered. Recovery was done by homogenisation of the leaf and plating the homogenate on selective medium. The number of bacteria recovered after 30 s was similar to the number recovered after 5 min, indicating rapid attachment of *L. monocytogenes* to leaf surfaces (Milillo et al., 2008).

In the second phase of binding, a strong irreversible attachment is thought to occur. This requires the use of physical or chemical energy to detach cells from a surface. Irreversible attachment involves bacterial cell surface appendages (Pratt & Kolter, 1998). Flagellar mediated motility, type I pili, type IV pili and curli fimbriae have all been shown in different studies to confer irreversible attachment to surfaces such as lettuce (Pratt & Kolter, 1998;

Berger et al., 2009). Bacterial properties, bacterial inoculum, exposure time, plant species and other external factors such as temperature all influence attachment. Temperature affects many genes in *L. monocytogenes*, resulting in changes to the bacterial cell surface and consequently cell attachment to surfaces (Gorski et al., 2003).

Attachment of *L. monocytogenes* to radish at temperatures between 10 and 37 °C has been investigated. Attachment at 37 °C was low at all exposure times tested and Gorski et al. (2003) suggested this was due to temperature regulation of physiological activities such as flagellar biosynthesis which is downregulated at 37 °C. Li et al. (2002) showed that mild heat treatments affect attachment and colonisation of *L. monocytogenes* on lettuce leaves. When lettuce was stored at both 5 and 15 °C, populations of *L. monocytogenes* on lettuce previously treated at 50°C were higher than lettuce treated at 20°C. Li et al. (2002) concluded that heat treatment helps the growth of *L. monocytogenes* during storage. The growth of *L. monocytogenes* on 9 cm^2 lettuce leaves was conducted at different temperatures (5, 10, 15, 20 and 25°C). The shortest lag phase and the fastest growth rate were observed at 20 °C (Koseki & Isobe, 2005b). Although low temperatures reduce the growth of *Listeria*, its ability to grow and survive at refrigeration temperatures (0.5–9.3°C) has been reported (Walker et al., 1990).

Outer membrane proteins and extracellular polysaccharides can have an influence on bacterial attachment and colonisation to plant surfaces (Romantschuk, 1992; Strom et al., 1993). Bae et al. (2013) suggested that *Listeria* cellulose binding protein (LCP) plays an important role in the micro-organism's attachment to a lettuce leaf surface. A mutant version lacking the LCP significantly lowered the attaching ability of *L. monocytogenes* to lettuce leaf surfaces. The percentage attachment by the wild-type strain (2.97% ± 0.37%) was significantly higher than that by the LCP mutant (0.3% ± 0.05%), ($P < 0.001$) after they were stored at 15 °C for 7 days.

These findings suggest that there is more research to do in the subject of how *L. monocytogenes* attaches to lettuce leaves. The effects of environmental stresses and bacterial attachment to specific sites on lettuce plants would be of interest as the mechanisms involved depend on many factors. The importance of each of the factors is still not clear. It is important to note that most studies have used artificial inoculation in the laboratory and this might be different from the conditions that exist in the field. More work needs to be done to help us fully understand the attachment and survival mechanisms of *L. monocytogenes* on lettuce leaves.

2.3 Biofilm formation of *L. monocytogenes* in fresh produce

The ability of *L. monocytogenes* to form biofilms in fresh produce has been reported (Botticella et al., 2013). Biofilm formation involves the production of extracellular polymeric matrix by micro-organisms and this acts as a protective mechanism under environmental stress. In a bacteria biofilm, the bacteria community attach to a surface and encase themselves within a polysaccharide coat. The production of the polymeric matrix gives the biofilm a complex structure. This mechanism protects bacteria from disinfectants and sanitizers (Niemira and Cooke, 2010). In the biofilm state, growth and maturation of *L. monocytogenes* occurs until it reaches a dispersion stage where detachment of cells occurs to colonise other surfaces (Zhao et al., 2017).

The occurrence of biofilms on leaf surfaces of fresh produce is minimal compared to biofilm formation on root surfaces (Hallman et al., 2001). The root zone secretes mucilage which is an excellent compound capable of supporting bacteria growth. Another reason might be the different environmental conditions experienced on the leafy parts of a plant such as changes in temperature and water availability (Yaron & Romling, 2014). Surfaces of leaves are covered with a hydrophobic cuticle which supports the attachment of hydrophobic molecules

(Fernández et al., 2017). It has been reported that disruption in the cuticle may release hydrophilic molecules such as juices from leaves which are attractive to pathogenic bacteria (Patel and Sharma, 2010). Pathogenic bacteria show preferential attachment to injured sites than to intact surfaces (Ells and Hansen, 2006). Structures on plant surfaces that have been associated with bacterial biofilm formation are the stomata, trichomes, veins and cell wall junctions (Yaron & Romling, 2014).

Surfaces of certain produce types have been reported to retain higher numbers of bacteria than others (Patel & Sharma, 2010). The attachment of *Salmonella enterica* to Romaine lettuce was significantly greater than attachment to Iceberg lettuce or cabbage (Patel & Sharma, 2010). Fresh produce surface properties such as roughness can be a possible factor influencing bacterial attachment (Wang et al., 2012). A study by Singh et al., (2018) found that the efficacy of Peracetic acid (PPA-100mg/L) to reduce *E. coli* O157:H7 on surfaces of fresh produce was dependent on surface properties of the produce. In their study, the reduction of *E. coli* O157:H7 was lower in produce with coarse surfaces (such as lettuce and cantaloupe) than produce with smooth surfaces (such as lemon and tomatoes). PAA (100 mg/L) reduced *E. coli* O157:H7 on lettuce by 2.2 log CFU/g. However, *E. coli* O157:H7 reduction of 5.5 log CFU/g was observed on tomato surfaces after PAA (100 mg/L) treatment (Singh et al., 2018). Many studies have investigated the ability of pathogenic bacteria to form biofilms on surfaces of processing equipment used for fresh produce (Kocot & Olszewska, 2017). The materials used in the fresh produce processing facilities such as stainless steel, polystyrene and glass have been identified as potential niches for biofilm growth (Ormanci & Yucel, 2017).

The role of certain genes in promoting or reducing the biofilm formation of pathogens on produce has been reported [Van &Abee, (2010); Barak et al., (2005)]. Van der Veen and Abee, (2010) demonstrated that SigB is important for *L. monocytogenes* biofilm formation as well as

resistance to disinfectants. A mutant version of SigB reduced the biofilm forming ability of *L. monocytogenes* and reduced its ability to resist disinfectants. In a different study, the *rpoS* gene was found to be important in the biofilm formation of *Salmonella enterica* in alfafa sprout (Barak et al., 2005). Since fresh produce are given minimal treatment without heating, an increase in the occurrence of biofilms on produce surfaces as well as produce processing equipment represents a food safety risk. In addition, the ability of biofilms to protect pathogens from disinfectants can exacerbate the risk of pathogen survival and growth. This necessitates the development of novel food control methods in the fresh produce industry.

2.4 Factors that affect colonisation of lettuce by *L. monocytogenes*

Attachment is a prerequisite for colonisation. Without attachment, *L. monocytogenes* cannot colonise lettuce leaves. Most of the factors that affect attachment also contribute to colonisation. Colonisation is the ability of the bacteria to grow and survive on the leaf surface. Specific properties related to the bacteria, specific features of the leaf as well as environmental conditions are essential to the understanding of a successful colonisation by *Listeria* on a leaf surface.

Flagella, outer membrane proteins and extracellular polysaccharides may influence bacterial colonisation of a leaf. Depending on the produce surface, *L. monocytogenes* uses its dynamic flagella mechanisms to successfully attach to different produce surfaces (Gorski et al., 2009). Flagellar motility is needed for *L. monocytogenes* to attach to fresh-cut radishes (Gorski et al., 2003). Tan et al. (2016) showed that pectin and xyloglucan, which are major structural components of the cell wall, help *Listeria* to attach to plant surfaces.

Other factors that have been demonstrated to affect colonisation are leaf age, leaf topography, architecture and leaf surface microflora. Leaf surfaces are generally not an ideal environment

for bacteria to obtain nutrients if compared to the nutrients they get from other foods such as meat and milk products (Fatima & Senthil-Kumar, 2015; Lindow & Brandl, 2003; Mercier & Lindow, 2000). Leaf age has been found to affect the growth of pathogens on leaf surfaces. The populations of *E. coli* O157:H7 inoculated on young lettuce leaves (tenth to twelfth leaves to emerge) was higher than old lettuce leaves (first and second leaves to emerge) after 48 h at 28°C. From an initial inoculum of 4 log CFU mL^{-1}, approximately 6 log CFU g^{-1} was found on young lettuce leaves while about 5.5 log CFU g^{-1} was found on old leaves. Pathogenic bacteria grew to higher numbers in exudates of young leaves than in exudates of old leaves suggesting that the high concentration of nitrogen and carbon in young leaves may account for their role in supporting pathogen growth (Brandl & Amundson, 2008). Young plants are not well resourced to protect themselves from foreign bacteria. This is due to the immaturity of protective defence structures such as casparian strip (a thick cell wall composed of suberin) and plasma membranes in young plants. Bacteria can have easy access to young plant tissue leading to their transport to inner cell tissues like the xylem (Warriner et al., 2003). Young lettuce leaves appear to have a greater risk of colonisation with pathogenic bacteria than older leaves.

Takeuchi et al. (2000) demonstrated that *L. monocytogenes* attach preferentially to the cut edge of lettuce, while *Pseudomonas fluorescens* showed greater attachment to the lettuce surface than the cut edge of lettuce. *L. monocytogenes* has a hydrophobic surface explaining the preferential attachment to the damaged lettuce tissues that lack a waxy cuticle (Mafu et al., 1991).

A study to investigate internalisation of *L. monocytogenes* in 20-day-old plants grown in a commercial potting mix was conducted by Shenoy et al. (2017). They found on average 3.9 cells of *L. monocytogenes* per mm^3 of plant tissue. Using a fluorescent microscope, they

observed *L. monocytogenes* being internalised in the xylem, cortex, pith and epidermis of lettuce tissues. The majority of *L. monocytogenes* was localised in the pith (17.3%), the innermost part of the stem (Shenoy et al., 2017). This suggests that internalised bacteria can move to other tissues in the plant. Other authors have also reported the ability of human pathogenic bacteria such as *E. coli* and *Salmonella* to internalise within lettuce tissues (Riggio et al., 2019; Gomes et al., 2009; Kroupitski et al., 2009).

The persistence of a bacterium on plants depends on its response to stresses from the environment as well as its ability to make use of minerals from the plant as nutrient source (Brandl, 2006). Biofilm formation can provide a shelter for the bacterium against non-conducive environments leading to a successful colonisation (Di Domenico et al., 2017; Aruscavage et al., 2006). Kroupitski et al. (2009) reported that light from a high-intensity bulb (100 μE m^{-2} s^{1}) can affect the colonisation of bacteria on iceberg lettuce. There was a localisation of *Salmonella* cells around stomatal cells and subsequent invasion into stomatal tissues when lettuce leaves were incubated with *gfp*-tagged *Salmonella enterica* in the presence of visible light. However, incubation in the dark led to poor attachment and poor stomatal colonisation (Kroupitski et al., 2009). This is suggestive of pathogens being attracted to plant cells which can carry out photosynthesis. Penetration of pathogens through the roots, wounds or the stomata is one of the main routes through which they use to internalise plant tissues (Kroupitski et al., 2009).

High relative humidity and very low oxygen conditions may also increase the risk of colonisation by pathogenic bacteria (Zoz et al., 2016; Al-Qadiri et al., 2015; Cantwell & Suslow, 2002). Enhanced wettability of leaf veins is another factor which helps colonisation of leaves by epiphytic bacteria (Leben, 1988). Warm incubation temperatures and the presence of

free water on leaves favour bacterial growth in the presence of nutrients. During warm weather, these conditions are likely to occur in lettuce fields (Charles-Edwards et al., 1987).

From the above discussion, it is evident that several factors affect the colonisation of lettuce by *L. monocytogenes*. Additional research prioritising the factors with the greatest effect will be important in devising methods to prevent colonisation of lettuce by *L. monocytogenes*.

2.5 Hydroponic growth system

Hydroponic production is the use of nutrient solutions to support plant growth without soil (Kratky, 2005). Hydroponic cultivation is usually done indoors such as in greenhouses (Touliatos et al., 2016). This is a controlled environment for vegetable production. The nutrients solution that is used to support plant growth is composed of beneficial nutrients in balanced quantities for plant use (Gent, 2003). Plants are grown in seed cups with a sterile support medium such as vermiculite. These are then placed in troughs which serve as support. There are two valves on the opposite sides of the trough. An inlet valve through which the nutrient solution gets to the plants and an outlet valve which controls the level of the nutrient solution in the system. A pump connected to PVC water pipes is needed to pump the nutrient solution up to the hydroponic trough. Through the perforations at the base of the seed cups, the roots of the plant reach the nutrient solution for the absorption of nutrients. For most hydroponic set-ups, especially the in-door ones, a light source is provided on top of the plants since plants need light for photosynthesis (Jones, 2014).

Proper maintenance of hydroponic set-ups is important for plant health. These include daily monitoring of the pH of the nutrient solution, a regular monitoring of the electrical conductivity (EC) of the nutrient solution and routine change of nutrient solution to ensure a clean set up. Maintenance of pH and EC are of vital importance to the growth of plants (Sambo et al., 2019).

The design of a well-maintained hydroponic set up prevents it from many potential contaminants. However, it has been reported that, contamination of the nutrient solution used for the hydroponic production of lettuce contaminated the lettuce (Wang et al., 2020). This might be possible due to the ability of pathogens to internalize in lettuce. This indicates that, any source of contamination in the hydroponic set up can spread to other parts of the hydroponic system. Figure 2.1 shows a set-up of lettuce grown in a hydroponic system.

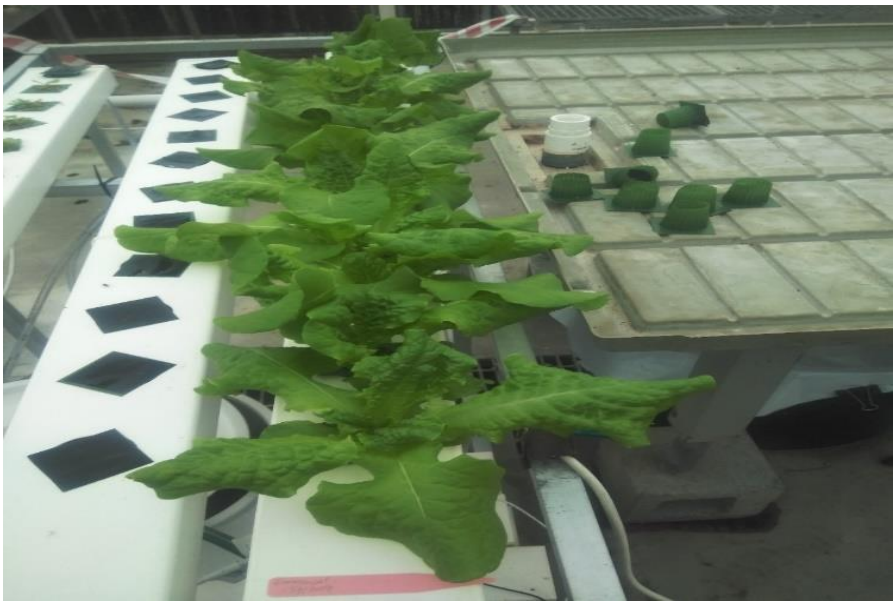

Figure 2.1. Lettuces grown in a hydroponic system.

2.6 Effects of different cultivation systems

Lettuce can be grown in the traditional (conventional), organic or hydroponic systems. Chemical fertilisers and/or composts are used in the traditional system, organic systems only use composts while the hydroponic system uses nutrient solutions to aid plant growth. Environmental control is easier to achieve when plants are grown hydroponically (Chaves et al., 2000). There are many potential sources of pathogenic bacterial contamination when fresh produce is grown traditionally or organically. These sources include soil, natural irrigation

water, manure and farm animals. The lack of these sources might be a reason why hydroponically grown lettuce is less likely to be contaminated with pathogenic bacteria such as *L. monocytogenes* (Neto et al., 2012).

Little research has been done on comparing counts of *L. monocytogenes* on conventionally, organically and hydroponically grown lettuce surfaces (Maffei et al., 2016). It has, however, been reported that hydroponic systems do not allow the growth of *L. monocytogenes* as the number of *L. monocytogenes* on lettuce grown in a hydroponic system did not change after 9 days (Jablasone et al., 2005). Carducci et al. (2015) reported the possibility of the spread of viral contamination of lettuce grown in a hydroponic system. This showed that contamination in one point of a hydroponic system can be transferred to other plants in the system. Thus, if *L. monocytogenes* contamination occurred it may be able to contaminate a whole hydroponic system.

Several studies have compared other bacteria on lettuces grown in different growth systems finding different results. The average mesophilic aerobic bacteria count in 25 g of iceberg lettuce grown with traditional cultivation was 6.48 log CFU g^{-1}, 6.85 log CFU g^{-1} for organic cultivation and 4.35 log CFU g^{-1} in a hydroponic system (Neto et al., 2012). Hydroponically grown lettuce had lower mesophilic aerobic (2.16 log CFU g^{-1}) and lactic acid (1.61 log CFU g^{-1}) bacteria counts than conventionally grown lettuce (4.93 and 2.86 log CFU g^{-1}) for mesophilic aerobic and lactic acid bacteria respectively (Lima et al., 2013).

A greater adherence of *Salmonella* Enteritidis to lettuce grown in a hydroponic system was recorded compared with conventionally grown lettuce. From an initial inoculum concentration of 7 log CFU mL^{-1}, they reported a 5.2 log CFU g^{-1} attachment to hydroponically grown lettuce while there was a 4.6 log CFU g^{-1} attachment to lettuce grown in a conventional system (Lima et al., 2013). Atomic force microscopy revealed that surfaces of hydroponically grown lettuce

leaves are rougher (1211 nm) than the conventionally grown ones (293 nm) (Lima et al., 2013). The surface roughness was analysed using the tapping mode technique taking into consideration the difference between the height and depth of the leaf surface. Hydroponically grown lettuce is more hydrophobic than the conventional grown lettuce. The free energy of interaction value for hydroponically grown lettuce was -15.43 mJ m^{-2} while that of the conventionally grown lettuce was 0.65 mJ m^{-2}. A negative free surface energy has been associated with stronger bacterial adherence (Lima et al., 2013).

Lettuce grown in a hydroponic system showed greater colonisation by *Salmonella* Typhimurium (5 log CFU g^{-1}) compared with a soil system (2.37 log CFU g^{-1}) however, *E. coli* O157:H7 was able to internalise in lettuce grown in soil but did not internalise in lettuce grown hydroponically (Franz et al., 2007). When lettuce was washed with sterile distilled water, the mesophilic aerobic count reduced 0.93, 0.80 and 0.78 log CFU g^{-1} in hydroponic, traditional and organic systems, respectively (Neto et al., 2012). This may be due to the more stringently controlled environment found in a commercial hydroponic system. Consequently, in a commercial hydroponic growing operation, the interactions with other microorganisms and nutrients that can be found in an organic or traditional cultivation could be more limited. The microorganisms or nutrients common in the traditional or organic system may aid in the growth and survival of the whole microbial community.

These examples suggest that growing lettuce in a hydroponic system can reduce bacterial contamination as it is a controlled system. However, a source of contamination in the system can readily spread throughout the hydroponic system (Carducci et al., 2015). Other reasons such as cost may be the reason why growing plants in a hydroponic system are less common. It will be interesting to know the effects of inherent properties of hydroponic grown lettuce on *L. monocytogenes* colonisation and the potential risks this brings to hydroponic growth lettuce.

2.7 Effects of different washing treatments

The practice of washing fresh produce with water has become part of our daily life. Washing with water reduces microbial load on fresh produce (Uhlig et al., 2017; Kilonzo-Nthenge et al., 2006). The flow rate of wash water affects the number of bacteria which can be removed since washing with a high flow rate (8L/min) results in a significant ($p < 0.05$) reduction of bacteria counts (Uhlig et al., 2017). In another study, washing spinach under running tap water with a flow rate of 2L/min was more effective than washing by water immersion. The number of *Salmonella* on spinach reduced by 1.52-1.62 log CFU/g when washed with running water but only 0.75-0.81 log CFU/g was reduced when washed by water immersion (Kilonzo-Nthenge & Liu, 2019). Richard & Cooper (1995) reported that the physical forces involved in washing affect the number of microorganisms removed from the plant surface. The addition of chlorine to wash water has been shown to reduce the population of pathogens on raw vegetables and has proved to be the most cost-effective way of lowering microbial load on plant material (Gil et al., 2009; Adams et al., 1989) but total elimination of attached pathogens cannot be assured (Kilonzo-Nthenge & Liu, 2019; Beuchat, 1998). Many fresh produce industries use chlorine concentrations of 20–200 ppm with pH values between 6.0 and 7.5 for 1–2 min to sanitise the water used in washing their produce to prevent cross-contamination (Fan & Sokorai, 2015; Suslow, 2001; Beuchat, 1998). Zhang & Faber (1996) observed that the reduction in *L. monocytogenes* on fresh-cut lettuce treated with 200 ppm chlorine (pH 8.43-9.31) for 10 min at 22 °C was 1.7 log CFU g^{-1}. In vitro experiments by Brackett (1987) showed that the action of chlorine against *L. monocytogenes* occurs primarily during the first 30 s of exposure. In a study by Fu et al., (2018), washing a batch of lettuce (800 g) in sterile tap water (3°C and pH 7.2) without chlorine treatment resulted in 3.51 log CFU/g *E. coli* O157:H7 reduction. However, under the same conditions, when washing was conducted with tap water with 20 ppm chlorine (pH 6.3), *E. coli* O157:H7 on lettuce reduced by 6.54 log CFU/g (Fu et al., 2018).

Lettuce washed with sodium hypochlorite (70 ppm, (mg L^{-1}), pH (6.40) for 2 min at 12.8 °C resulted in more than 2 log reduction in the number of aerobic bacteria (Soriano et al., 2000).

Several studies have also reported the inefficiency of chlorine as a sanitiser for fresh produce (Olmez & Kretzschmar, 2009; Tomas-Callejas et al., 2012). The inefficiency of chlorine may be partly due to lack of optimised sanitising potential conditions for chlorine such as pH. Failure to adjust pH to effective sanitising levels will result in inefficiency in reducing microbial loads (Sapers, 2001). Organic matter may also inhibit the efficiency of chlorine when used as a sanitiser. A chlorine concentration of 200 ppm with a pH of 6.5 could not remove *L. monocytogenes* on spinach surface after washing for 1 min at room temperature. At least 4.86 log CFU g^{-1} of *L. monocytogenes* remained on spinach surfaces after an initial inoculum of 6 log CFU g^{-1} (Ijabadeniyi et al., 2011). Ryu & Beuchat (2005) reported that after a 10 min treatment with 10 ppm of chlorine pH (7.4) cell numbers of *E. coli* O157:H7 strain 43895-EPS planktonic cells grown at 22 °C decreased from 8.9 to 4.3 log CFU mL^{-1}. Organic substances released from a lettuce plant after it has been cut can negatively affect the efficiency of chlorine as a sanitiser for fresh produce (Nou & Luo, 2010).

Baert et al. (2009) observed a 0.61 log CFU g^{-1} average reduction in numbers of *L. monocytogenes* inoculated on 4 cm^2 shredded iceberg lettuce when they were washed for 5 min with 200 mg L^{-1} sodium hypochlorite with a pH of 5.90–5.95. However, with the same conditions, there was no significant decline in *L. monocytogenes* numbers when they were washed with 20 mg L^{-1} sodium hypochlorite. Antimicrobial activity of chlorinated compounds such as sodium hypochlorite depends on the amount of free available chlorine that is exposed to microbial cells (Beuchat, 1998). The amount of free available chlorine in 200 mg L^{-1} was found to be 112 mg L^{-1} whereas 20 mg L^{-1} contained 0.5 mg L^{-1} free available chlorine (Baert et al., 2009). This explains the efficacy of a higher concentration of sodium hypochlorite (200

mg L^{-1}) in reducing *L. monocytogenes* cells than a lower concentration of 20 mg L^{-1}. Chlorinated water is an effective strategy for controlling cross-contamination during processing if recommended free chlorine levels are maintained in the process water (Chen & Hung, 2018; Beuchat, 1998).

Other washing techniques such as acetic acid have been used with water to wash fresh produce. Neto et al. (2012) tested the efficiency of acetic acid (1%) and sodium hypochlorite (150 mg L^{-1}) as a sanitiser on lettuce. After washing lettuce for 15 min, sodium hypochlorite reduced aerobic bacterial counts from 6.48 log CFU g^{-1} to 5.38 log CFU g^{-1} for traditionally grown lettuce, 6.85 to 5.55 log CFU g^{-1} for organically grown lettuce and 4.35 to 3.53 log CFU g^{-1} for hydroponically grown lettuce. Acetic acid caused a reduction of 4.88, 4.63 and 3.22 log CFU g^{-1} in traditional, organic and hydroponic systems respectively (Neto et al., 2012). Chlorine dioxide gas (5 ppm) reduced *L. monocytogenes* on shredded lettuce by 5.9 log CFU g^{-1} after 5 min treatment at 21 °C (Rodgers et al., 2004).

Several studies have used ozone as a sanitiser for fresh produce (Aslam et al., 2019; Beuchat, 1998; Kim et al., 1999). Ozone (3 ppm) reduced *L. monocytogenes* on shredded lettuce by 6.0 log CFU g^{-1} after 5 min treatment at 21 °C (Rodgers et al., 2004). Washing of lettuce before cutting is more advisable than after cutting in terms of microbial reduction. This may be due to the exudate interfering with sanitisers (Nou & Luo, 2010). There was only a 1.1 log CFU mL^{-1} reduction in *E. coli* O157:H7 after fresh-cut romaine lettuce was washed in chlorine water whereas washing whole lettuce before cutting resulted in a reduction of 1.9 log CFU mL^{-1} (Nou & Luo, 2010). During washing of fresh produce, the wash water to produce ratio can affect the efficacy of washing. The quality of water (in terms of total dissolved solutes) decreased from 520.0 to 719 mg L^{-1} as the amount of lettuce was changed from 2 to 18 kg (Luo, 2007).

A study conducted by Gao et al., (2020) found that the amount of lettuce washed in a batch affects the risk of cross contamination. The first batch of 24 red leaf lettuce heads inoculated with 5 log CFU/g *L. monocytogenes* washed with 76 L of tap water mixed with antimicrobials significantly contaminated non-inoculated lettuce during the second batch of washing. However, the cross-contamination effect was insignificant in the second batch of washing when only 8 red leaf lettuce heads were used in the first batch. This implies that, retailers should consider the product volume during washing of fresh produce as it can affect produce contamination (Gao et al., 2020).

Allende et al. (2008) reported that an initial *E. coli* concentration of 5.1 log CFU g^{-1} was able to contaminate uninoculated escarole through the wash water. However, a 3.2 log CFU g^{-1} was not able to be detected on the escarole (Allende et al., 2008). This suggests that the amount of contamination from the produce can affect water quality used for washing.

The ability of *L. monocytogenes* to internalize in lettuces (Shenoy et al., 2017) indicates that surface washing may not have any effect on the reduction of the pathogen. This is an important reason for the development of novel control methods in which the stress response is been generated from polyphenols in the leaves.

A combination of two different wash methods is sometimes more effective than just a single method (Lippman et al., 2020; Kumar & Ravishankar, 2019; Parish et al., 2003). A combined treatment of ozonized water (0.01-0.1 mg O_3/L) and 5% olive extract wash for 120 min resulted in 4.2 log CFU/g reduction in *Salmonella* Newport on iceberg lettuce. However, the use of only ozonised water (0.01-0.1 mg O_3/L) wash for 120 min only resulted in 2.1 log CFU/g *Salmonella* Newport reduction on iceberg lettuce (Kumar & Ravishankar, 2019). A reduction of 3 log was recorded in the population of coliforms when an Ozone-Tsunami [20 mg L^{-1} Ozone pH (7.50) and 300 mg L^{-1} Tsunami pH (3.79)] wash was used. Tsunami is a trade name which contains a

mixture of acetic acid (30–60%), peroxyacetic acid (15.2%) and hydrogen peroxide (11.2%) (Beltran et al., 2005). The inefficiency of traditional washing methods in the produce industry has been reported (Goodburn & Wallace, 2013). Control strategies in washing of fresh produce in the industries are insufficient to adequately protect public health. Additional research is needed to improve pathogen reduction during fresh produce processing.

2.8 Effects of different radiation treatments as alternative treatments for control

Overall, the microbial reduction that can be achieved with chlorine washes on lettuce and other leafy greens is in the range of 1–2 log CFU g^{-1} (Koseki et al., 2004; Virto et al., 2005). New control strategies for lowering the risk of pathogens on fresh produce are being devised. Recently, there has been an increase in the use of different forms of radiation as alternative treatments for pathogen control in fresh produce. These forms of radiation include the use of gamma radiation, light-emitting diodes (LED), ionizing radiation and UV-radiation (Berrios-Rodriguez et al., 2020; Josewin et al., 2018). The efficiency of the radiation treatment depends on the wavelength of the radiation, time of treatment, relative humidity and temperature. Higher doses of radiation results in greater microbial reduction, however, higher doses can adversely affect the quality of produce (Mahajan et al., 2014).

The use of LED to inactivate pathogenic bacteria has been reported. Josewin et al., (2018) used LED of wavelength 460 nm at a total dose of 5356 J/cm^2 to reduce *L. monocytogenes* population on cantaloupe rinds by 2.7 log CFU/g after 48 h at 4°C. Several studies on the use of ionizing radiation to control pathogenic bacteria on fresh produce have also been reported (Bidawid et al., 2000). The use of ionising radiation at specific doses (up to 4 kGy) on lettuce has been approved by the FDA (FDA, 2009). Gamma radiation (0.5kGy) treatment on tomatoes

inoculated with *L. monocytogenes* reduced the number of *L. monocytogenes* on tomato surfaces by 2.6 log after 7 days at 5°C (Berrios-Rodriguez et al., 2020).

Treating *L. monocytogenes* on iceberg lettuce leaf with 0.1 kGy X-ray at 22°C resulted in a 1.6 log CFU cm^{-2} reduction. Increasing the radiation dosage to 1.0 kGy X-ray under the same conditions reduced *L. monocytogenes* on lettuce by 4.1 log CFU cm^{-2}. A further increase to 2.0 kGy reduced *L. monocytogenes* on lettuce by more than 5 log CFU cm^{-2} (Mahmoud, 2010a). Mahmoud (2010a) reported no significant effect in the colour of lettuce when a 2.0 kGy radiation was used. There are some concerns about the use of irradiation in the food industry (Caputo, 2020). Food Standards Australia-New Zealand (FSANZ) has approved the use of irradiation for only a small number of fruits and vegetables such as tomato and mango for research purposes (MPI, 2015). Regulations by government agencies limit the use of the application of irradiation technology in the food industry (Caputo, 2020). Additional research and education are needed to explain the benefits of appropriate irradiation techniques to the stakeholders involved.

The population of psychrotrophic bacteria on lettuce leaves reduced from 4 log CFU g^{-1} to 2.1 log CFU g^{-1} after treatment with 0.1 kGy radiation (Mahmoud, 2010a). In a similar study, 0.75 kGy Xray at 22°C with 55-60% relative humidity reduced *L. monocytogenes* on tomato by 2.3 log CFU g^{-1}. Psychrotrophic bacteria counts on tomato surfaces were reduced from 4.7 to 3.0 log CFU g^{-1} after 0.1 kGy X-ray treatment (Mahmoud, 2010b).

Control of microorganisms on fresh produce by treatment with UV radiation has also been reported in the literature (Adhikari et al., 2015). UV is cheap to use, it maintains product quality and, unlike chlorine, it leaves no chemical residues on produce surfaces. Factors that are important in the efficacy of UV treatment are the wavelength, time of exposure, temperature, distance of the UV light source from the produce and intensity (power) of the UV light source

(Kim et al., 2013). UV wavelengths between 200 and 280 nm have often been used in pathogen control on fresh produce (Bintsis et al., 2000). When 25 g of lettuce contaminated with *L. monocytogenes* was exposed to 254 nm UV radiation (3.40 mW cm^{-2}) at 4 °C for 1 min, there was a 1.16 log CFU g^{-1} reduction in *L. monocytogenes*. A greater reduction of 2.12 log CFU g^{-1} was achieved at 25°C (Kim et al., 2013). This indicates that temperature affects the efficiency of UV treatments in fresh produce microbial contamination.

The closeness of the UV light source to fresh produce for microbial control has been studied. A 10 cm distance between the UV light and the sample was more effective than a 50 cm distance in reducing microbial load by >1 log CFU g^{-1} (Kim et al., 2013). Increasing the distance between the UV light source and fresh produce will reduce the intensity of the light on the plant surface. Higher UV intensities have been reported as being more effective in reducing microbial populations on fresh produce (Kim et al., 2013).

UV radiation treatment (11.9 kJ m^{-2}) with equal inoculum concentration of *L. monocytogenes* on different vegetables at 23°C showed variable results. There were reductions in apple (1.6 log CFU g^{-1}) and pear (1.7 log CFU g^{-1}). However, cantaloupe and strawberry only had 1.0 log CFU g^{-1} reduction (Adhikari et al., 2015). This indicates that surface roughness affects UV treatment efficacy. Pear and apple have smooth surfaces unlike strawberry and cantaloupe with rough surfaces. Fruit surface properties such as roughness, hydrophobicity and the presence of trichomes, hair-like outgrowths from epidermis of plants, and naturally occurring crevices on fruits such as cantaloupe can reduce the effectiveness of microbial control (Syamaladevi et al., 2013). UV radiation has also been used in combination with other control methods to reduce bacterial populations on fresh produce. A UV dose of 8 kJ m^{-2} after coating broccoli samples with chitosan resulted in a 1 log CFU g^{-1} reduction in *L. monocytogenes* (Severino et al., 2014).

Gamage et al. (2016) reported that UV-C (2.6 and 5.2 kJ m^{-2}) treatment induced the inhibition of growth of *L. monocytogenes* in extracts from broccoli. Treatment of fresh-cut broccoli branchlets with 2.6 kJ m^{-2} reduced the amount of *L. monocytogenes* on broccoli after 18 h from an optical density of 0.65–0.5 whereas a high dosage of 5.2 kJ m^{-2} after 18 h reduced *L. monocytogenes* from an optical density (600 nm) of 0.65–0.4. This confirms that high dosages of UV-C treatment can be more effective than lower doses in reducing *L. monocytogenes* on fresh produce. Mild stress responses from plants such as exudates released after an exposure to stress may be important in devising control strategies for pathogenic bacteria on fresh produce. These studies show the potential importance of UV-C in controlling pathogenic bacteria on fresh produce during pre or post-harvest processing. Many fresh produce industries do not use UV and other radiation due to the cost, maintenance and lack of technical knowledge. A summary of intervention steps, both chemical and irradiation, is given in Table 2.2.

Table 2.2. Efficacy of different intervention steps in controlling *L. monocytogenes* on lettuce

Lettuce variety	Intervention step	Conditions used	Reduction achieved	References
Fresh-cut lettuce	200 ppm Chlorine	pH 9.31 at 22°C for 10 min	1.7 log CFU g^{-1}	Zhang & Farber (1996)
Lettuce	70 ppm Sodium hypochlorite	pH 6.40 at 12.8°C for 2 min	More than 2.0 log	Soriano et al. (2000)
Shredded iceberg lettuce	200 ppm Sodium chlorite	pH 5.90 for 5 min	0.61 log CFU g^{-1}	Baert et al. (2009)
Shredded lettuce	3 pm Ozone	21°C for 5 min	6.0 log	Rodgers et al. (2004)
Iceberg lettuce	0.1 kGy X-ray	22°C	1.6 log CFU cm^{-2}	Mahmoud (2010a,b)
Iceberg lettuce	1.0 kGy X-ray	22°C	4.1 log CFU cm^{-2}	Mahmoud (2010a,b)
Iceberg lettuce	2.0 kGy X-ray	22°C	More than 5 log	Mahmoud (2010a,b)
Lettuce	254 nm UV radiation	3.40 mW cm^{-2} at 4°C for 1 min	1.16 log CFU g^{-1}	Kim et al. (2013)
Lettuce	254 nm UV radiation	3.40 mW cm^{-2} at 25°C for 1 min	2.12 log CFU g^{-1}	Kim et al. (2013)

2.9 Application of UV radiation in fresh produce safety

In recent years, there has been an increase in the use of UV radiation as a food control method since it has been found to be very effective in reducing bacteria population on fresh produce (Moriarty et al., 2018; Charles & Arul, 2007). The use of UV radiation involves less energy and cost compared to the other food control methods. Moreover, it is ecologically safe to use since no by products are produced (Koutchma, 2019). Due to these advantages, the application of UV has been endorsed as a food control method by the FDA (FDA, 2000).

UV radiation has been divided into three main parts; UV-A, UV-B and UV-C. The differences in these 3 types of UV radiation depends on the wavelength it emits. UV-A produces the highest wavelength which ranges between 315-400 nm. The wavelength of UV-B ranges from 280-315 nm and that of UV-C ranges from 200-280 nm (Rafieepour et al., 2015). Due to the harmful effects of UV-A and UV-B such as burning of skin and causing cancer (Trakatelli et al., 2007), UV-C is the most commonly use in the food industry to reduce bacterial levels. Another important characteristic of UV-C irradiation is its ability to preserve fresh produce to maintain produce quality and other sensory characteristics after exposure (Koutchma, 2019).

Upon an exposure of bacteria to UV radiation, the bacteria DNA absorbs the radiation which eventually leads to the disruption of the DNA structure (Rastogi et al., 2010). Microbial factors which affect the efficiency of UV radiation on microbial reduction include dosage, type of bacteria strain, treatment temperature, biofilm association and a DNA repair mechanism (Jones et al., 2016).

There have been several reports about the use of UV-C in reducing bacteria numbers on fresh produce. A UV-C dose of 0.30 J/cm^2 per pulse reduced the biofilm cell number of *L. monocytogenes* by 0.6-2.2 log CFU/ml on lettuce surfaces (Montgomery & Banerjee, 2015).

There was more than a 2 log reduction in *Salmonella* numbers in lettuce treated with a UV-C radiation of 150, 450 and 900 mJ/cm^2 (Ge et al., 2013).

A study by Martinez-Hernandez et al (2015) found *L. monocytogenes* as the most resistant pathogen against UV-C radiation compared to *E. coli* and *Salmonella* Enteritidis. In the study, a UV dose of 1.07 kJ m^{-2} was able to reduce 1 log of *E. coli* on the surface of broccoli. A UV-C dose of 0.02 kJ m^{-2} reduced *Salmonella* Enteritidis number by 1 log. However, for the removal of 1 log of *L. monocytogenes* on broccoli surface, a UV-C dose of 9.26 kJ m^{-2} was applied.

The resistance of a mixed-culture biofilm formed on lettuce surface to UV-C radiation was significantly higher than a mono-culture biofilm (Jahid et al., 2014). Bacteria residing on fruits with smoother surfaces such as apple are more likely to be affected by UV treatment compared to fruits with rough surfaces. A UV-C treatment of 0.223 kJ m^{-2} was able to reduce *Salmonella* numbers by 3.2 log on a tomato surface.

There are other studies about the use of UV radiation in combination with other methods to reduce pathogen counts on fresh produce (Collazo et al., 2019; Park et al., 2018). The use of water assisted UV-C treatment (a combination of water and UV-C) reduced *L. monocytogenes* by 2.1 log (Collazo et al., 2019). A combined treatment of UV-C and Chlorine dioxide gas (ClO_2-5ppmv) reduced *L. monocytogenes* by 3.14 log on spinach leaves after 20 mins. However, with the same conditions, single treatments of spinach leaves with only UV-C and ClO_2 gas (5ppmv) reduced *L. monocytogenes* by only 1.87 and 1.58 log respectively (Park et al., 2018). These examples above demonstrate the potential of UV radiation as well as combination of UV with other control methods in reducing pathogen contamination in the fresh produce industry. Additional research about pathogen control by UV radiation especially UV stress (which does not have any adverse effect on produce sensory qualities) might potentially

lead to discovering novel approaches important for fresh produce safety. UV treatments have focused on treating the produce already contaminated with bacteria. There is the opportunity to investigate the effect of UV stress on preventing lettuce colonisation.

2.10 Effects of leaf surface microbiota as a control

Leafy greens such as lettuce have been shown to harbour diverse populations of bacteria (Yu et al., 2018; Truchado et al., 2017). Specific studies have revealed the effects of seasonal differences in lettuce microbiota (Yu et al., 2018), effect of different irrigation types on lettuce microbiota (Williams et al., 2013), effect of climatic conditions such as solar radiation exposure on lettuce microbiota (Truchado et al., 2017), effect of the location where lettuce is grown on lettuce microbiota (indoors, field grown or grown in the laboratory) (Williams & Marco, 2014) and other factors (Medina-Martinez et al., 2015).

Other researchers have also investigated the interaction between indigenous microbiota found on leaf surfaces and pathogenic bacteria. In a study by Carlin et al. (1996), there was a significant growth of *L. monocytogenes* inoculated onto disinfected endive leaves. Disinfection of endive leaves reduced indigenous microbiota such as *Pseudomonas* found on endive leaf surfaces. *L. monocytogenes* could not achieve a significant growth on non-disinfected endive leaves due to inhibition by *Pseudomonas* (Carlin et al., 1996). In another study by Wan et al., (2017), a change in the tomato's rhizosphere bacterial community was observed after inoculation of *Bacillus amyloliquefacians* (*B. amyloliquefacians*). This caused the bacterial community to supress *Fusarium oxysporum* (*F. oxysporum*) (the pathogen which causes tomato wilt). After inoculating tomato with *B. amyloliquefacians*, analysis after 10 days revealed an increase in *Pseudomonas* (indigenous to tomato), thereby inhibiting the growth of *F.*

oxysporum (Wan et al., 2017). The ability for *Pseudomonas* to suppress pathogen growth has been attributed to competition for nutrients (Wei et al., 2006).

In another study, the ability of the native microbiota of cantaloupe to reduce *L. monocytogenes* growth was reported by Ukuku et al., (2004). They found that sanitizing cantaloupes with 200 ppm chlorine significantly ($p < 0.05$) reduced surface native microbiota (which included lactic acid bacteria and *Pseudomonas* spp). The attachment of *L. monocytogenes* to cantaloupe surfaces was significantly higher for the chlorine sanitized cantaloupes than the non-sanitized cantaloupes. They observed that, a reduction of the native microbiota, due to chlorine treatment, gives way to rapid attachment and growth of *L. monocytogenes* (Ukuku et al., 2004).

Inoculation of lettuce with *Pseudomonas* under field conditions caused an increase in the population of *Acidobacteria* and a decrease in the number of *Proteobacteria*. This caused the pH of lettuce to decrease and a decrease in lettuce pH has been associated with a reduction in the growth of certain plant pathogens (Cipriano et al., 2016; Dutta et al., 2014). Wei et al., (2006) investigated the effect of indigenous microbiota (*Pseudomonas putida-P. putida*) isolated from iceberg lettuce on *L. innocua*. They found that *P. putida* (an isolate native to iceberg lettuce) decreased the *L. innocua* population to levels below the detection limit (<100 CFU/g) after 8 days at 4°C. They attributed *P. putida*'s inhibition of *L. innocua* to its competitive ability, often referred to as competitive exclusion. They suggested that, a culture of *P. putida* (which poses no safety risk as well as no adverse effect on the sensory qualities of lettuce) can be used in lettuce processing lines before the final wash (Wei et al., 2006).

Other studies have also investigated the interactions between resident bacteria isolated from the food processing environment and *L. monocytogenes* (Carpentier & Chassaing, 2004). *Bacillus* species CCL9 (an isolate from a food processing environment) reduced *L. monocytogenes* biofilms formed on stainless steel by 3 log (Carpentier & Chassaing, 2004). In

a similar study, *Staphylococcus sciuri* (an isolate from a food processing environment) reduced *L. monocytogenes* biofilms on stainless steel by 0.9-2.7 log after 4 days (Leriche & Carpentier, 2001).

These studies suggest the potential for indigenous fresh produce and fresh produce processing environment microbiota to prevent colonisation and reduce the survival of foodborne pathogens. The effect of sanitization of fresh produce can lead to reduction in resident microbiota, however, contamination with pathogens after sanitization can result in a better growth of pathogens since pathogens will likely face less competition for space and nutrients. Additional research about the use of native leaf surface microbiota (which do not have any food safety risk as well as maintaining the sensory qualities of the fresh produce) will be very important for the fresh produce industry.

2.11 Strategies that can be used to minimise colonisation of lettuce by *Listeria* to enhance food safety

L. monocytogenes control on lettuce has become a challenge because of the inefficiency of current control methods (Murray et al., 2017). The ability of *L. monocytogenes* to thrive and adapt to different environmental conditions also makes it difficult to control (NicAogain & O'Byrne, 2016). It is impossible for a vegetable processing facility to test all individual products for microbial contamination due to lack of resources, time and labour and the destructive nature of current microbial test methods. Protocols for testing pathogenic contaminants are specific to different pathogens and expensive, making large scale identification of pathogens unrealistic. Food regulatory agencies in some countries such as the United States, have established statistically valid sampling regimes used for testing the safety of fresh produce (National Advisory Committee on Microbiological Criteria for Foods, 1999). Another approach is testing for microorganisms, such as *E. coli*, that may serve as indicators

of pathogen contamination. However, to the best of our knowledge, there has not been any report relating to fresh produce using enteric indicators for the presence of *L. monocytogenes*. Burnett et al. (2020) found a positive correlation between aerobic plate counts (APC) of samples collected from surfaces in retail grocery produce and *L. monocytogenes* prevalence. Additional research is needed to understand enteric indicator tests in fresh produce.

The best control method is prevention of pathogen contamination by eliminating the source. Contamination from the soil, manure and activities of animals in the environment (Iwu & Okoh, 2019) of lettuces grown in fields is hard to control. This is exacerbated by the weather with the likelihood of pathogen spread and growth on the lettuce leaves increasing in wet conditions (Machado-Moreira et al., 2019). Animal activity in lettuce fields should be controlled and untreated animal manure should not be used. Workers in the field should strictly adhere to good hygienic practices. Irrigation water sources for lettuce cultivation should routinely be checked to make sure they are free from pathogenic microorganisms (Dao et al., 2018). Hydroponic systems are a more hygienic method of growing lettuce than field systems (Manzocco et al., 2011). A greater use of hydroponics and farmer education on hygienic growing systems would be beneficial to the industry. Processing equipment should be regularly controlled and checked. Controlling the sanitiser concentration, exposure time and pH is critical to optimising lettuce processing. Using more than one treatment system (combination treatments) may be more effective than a single treatment (Lippman et al., 2020). Some substances produced by plants can also serve as natural control measures for pathogenic bacteria (Kang & Song, 2017). The population of *L. monocytogenes* on leafy salads reduced by 1.96-2.97 log CFU/g after treatment with 7mg/ml pomegranate pomace extract (Kang & Song, 2017). Carrots have been reported to inhibit proliferation of *L. monocytogenes* (Beuchat & Brackett, 1990b). This is a more novel approach that has not been applied to routine lettuce processing and additional investigations into this will be important to the produce industry.

2.12 Conclusion

An increase demand for lettuce will arguably lead to greater volumes of produce entering the processing chain, and therefore, the risk of food pathogen contamination in the future will be ever-present. Processes used in lettuce preparation such as cutting, shredding and washing can easily spread the source of contamination to lettuce leaves. The current control steps such as washing and radiation treatments are able to reduce *L. monocytogenes* colonisation to some extent. However, these control steps have limitations as both bacterial properties and the condition of the lettuce plants influence the efficacy of these control measures. Regular testing for microbial quality on fresh produce sold at retail is important to ensure they are safe for consumption. Reinforcement of good agricultural practices, good hygienic practices, good manufacturing practices and good storage practices are essential in the control of *L. monocytogenes* on fresh lettuce. Investigations into novel control methods focusing on qualities potentially capable of affecting lettuce such as hydroponic growth systems and UV stress may lead to new discoveries which may enhance fresh produce safety. In order to develop effective control measures, it is important to identify the differences between hydroponic and soil grown lettuce and understand how the differences (if any) can affect *L. monocytogenes* survival. A comparison between hydroponic and soil grown lettuce with respect to *L. monocytogenes* attachment, survival and biofilm formation have not been studied. Studies on the effect of UV stress on *L. monocytogenes* populations on lettuces have not been carried out.

2.13 References

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CHAPTER 3 Prevalence of *Listeria monocytogenes* contamination in lettuces sold in supermarkets

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Abstract

The popularity of bagged salads sold has increased due to their ease of transportation and convenience but there are food safety risks. In this study, the prevalence of *L. monocytogenes* in 100 samples of ready-to-eat bagged and non-bagged lettuces sold in supermarkets in New Zealand was examined. Five samples tested positive for *L. monocytogenes* and two further bagged lettuce samples tested positive for other *Listeria* species. None of the un-bagged lettuce samples were contaminated with *Listeria*. The results of this survey indicate a higher microbial risk associated with consumption of bagged salads. This underlines the need for the fresh produce industry to ensure efficient sanitization of their produce before bagging to reduce the risk of *Listeria* contamination.

3.1 Introduction

Listeria monocytogenes (*L. monocytogenes*) is a Gram-positive bacterium which has been implicated in many outbreaks of food borne illness related to fresh produce (NSW Food Authority 2018; Self et al., 2019) including lettuce (CDC, 2016) and packaged salads (Self et al., 2019). In recent years, foodborne outbreaks and recalls associated with fresh produce have also been reported in New Zealand. LeaderBrand (the biggest salad producer in New Zealand) recalled all its products due to lettuce contaminated with *L. monocytogenes* in 2017 (MPI, 2017). Alfalfa sprouts produced by Golden State Foods (GSF) fresh New Zealand were contaminated with *Salmonella* Typhimurium phage type 108/170 and this caused sickness in about 70 people in 2019 (Food Safety News, 2019). *L. monocytogenes* causes listeriosis, an infection with symptoms such as fever, headache and diarrhoea. Under severe cases, listeriosis can cause septicaemia, meningitis and miscarriage in pregnant women. Older adults, pregnant women and people with weak immune systems have a greater chance than the general population, of getting listeriosis (Buchanan et al., 2017). According to the Australian and New Zealand guidelines for fresh produce safety, regular testing for the microbial quality of produce is important since it is a useful tool to verify practices used in preventing produce contamination (FPSC, 2019).

The sales of bagged salads have been increasing (Norris, 2018; Tan, 2018) which might be due to the ease of transportation as well as convenience. Customers might refrain from purchasing un-bagged salads in supermarkets due to the perceived risk of them being contaminated. For instance, consumers who come to supermarkets might have contaminants on their hands which can contaminate un-bagged salads after touching. Un-bagged salads can also be exposed to insect pests (Olaimat & Holley, 2012). This has led to many produce processing companies bagging their products and hence giving rise to more bagged salads available in supermarkets.

The fresh produce industry uses different types of packaging to prolong the shelf life of produce and reduce microbial contamination (Putnik et al., 2017). This may involve creating a modified atmosphere by decreasing the concentration of oxygen and increasing the concentration of carbon dioxide (Manolopoulou et al., 2010). Modified atmospheric packaging (MAP) is used to adjust the composition of air surrounding the produce. The use of MAP in fresh produce such as lettuce, kiwifruit and apples has been used in New Zealand for more than a decade (Atiken et al., 2004). Some studies have reported the inability of MAP to prevent the growth of pathogens (Carrasco et al., 2008). *L. monocytogenes* increased by 3 log CFU/g on lettuce packaged in 4.65-6.2% CO_2, 2.1-4.3% O_2 and a balance of N_2 MAP after 7 days at 13°C (Carrasco et al., 2008).

The microbial quality of open and packaged salads has been reported in several studies (Sant'Ana et al., 2012; Sagoo et al., 2003a) with samples including other leafy greens apart from lettuce (Sant'Ana et al., 2012). To the best of my knowledge, no studies have been done to specifically compare *L. monocytogenes* contamination on bagged and un-bagged lettuces sold in supermarkets. The objective of this study was therefore to evaluate the prevalence of *L. monocytogenes* in bagged and un-bagged ready-to-eat (RTE) lettuces sold in supermarkets in a major city in New Zealand. The results that will be obtained from this survey will be important to justify maintaining a proper fresh produce control program in New Zealand preventing future *L. monocytogenes* related outbreaks and recalls.

3.2 Materials and Methods

3.2.1 Sample Collection and preparation

A total of 100 lettuce samples were collected for this survey over a period of 5 weeks (designated weeks I, II, III, IV and V) in September and October 2019. Bagged and un-bagged

lettuce samples were purchased from two supermarkets in Palmerston North (a major city in NZ); Supermarket A and Supermarket B. These two supermarkets represent the main fresh produce distribution chains in New Zealand. Fifty samples were collected from each supermarket with 30 bagged lettuce and 20 bagless lettuce samples. The temperature (recorded by a Testo food safety thermometer (Testo 104-IR, NZ) of the produce section in the supermarkets on each collection day was between 4 and 7°C. To ensure representative sampling for lettuce samples, different varieties of lettuce with the same production and 'used before' dates were randomly selected at each sampling time. Only lettuce samples with 3-4 days before their 'used before date' were collected. The weights of the bagged lettuce varieties sold in both supermarkets were either 100 g or 200 g. For the un-bagged lettuces, a whole head of lettuce was considered as one sample. Some lettuces sold in the supermarket at the time of collection were in open bags (not sealed) and they were also classified as un-bagged lettuce samples. Twenty lettuce samples were collected every week with 10 samples collected from each supermarket. Since there were more bagged lettuce varieties than un-bagged lettuce varieties, six bagged lettuce and four un-bagged lettuce samples were collected from each supermarket on each occasion. The same varieties were collected for all five weeks. Lettuce samples were aseptically transported to the laboratory in insulated ice chest containers. For the un-bagged lettuce samples, the outer leaves as well as any loose or discoloured leaves were discarded. Before microbial analysis, all lettuces were separately rinsed under a running tap with sterilized distilled water for 1 min to remove any surface contaminants. Microbial analysis was performed within 3 h of sample collection. The descriptions of the lettuce sample types collected are given in Table 3.1.

Table 3.1. Supermarket descriptions of lettuce samples

Supermarket A				Supermarket B			
Bagged Samples	**Sample Code**	**Bagless**	**Sample Code**	**Bagged**	**Sample Code**	**Bagless**	**Sample Code**
Brand 1 Baby leaf lettuce	1BLL	Brand 3 fancy green lettuce	3FGL	Bagged iceberg lettuce	BIL	Brand 5 lettuce traditional iceberg	5LTI
Brand 2 Baby leaf lettuce	2BLL	Brand 3 fresh cut lettuce	3FCL	Bagged baby cos	BBC	Brand 5 lettuce coral green	5LCG
Brand 2 baby cos lettuce salad	2CLS	Brand 3 cos lettuce	3CL	Brand 4 lettuce cos sweet petite	4LCSP	Brand 5 lettuce cos	5LC
Brand 2 Baby leaf salad	2BLS	Brand 3 mixed loose-leaf salads	3MLLS	Brand 5 lettuce green baby cos	5LGBC	Brand 5 lettuce twin pack coral	5LTPC
Brand 2 green & red cos salad mix	2GRCSM			Brand 5 lettuce buddies	5LB		
Brand 3 bagged iceberg lettuce	3BIL			Brand 6 lettuce shredded prepacked	6LSP		

3.2.2 Detection of *Listeria*

Microbial analysis of lettuce samples was done without enrichment and with enrichment. Twenty-five g of lettuce from each sample was homogenised with 225 mL of 0.1% peptone water (GranuCult®, Merck, KGaA, Germany) using a Smasher™ Lab Blender (AES-Chemunix) for 120 s at a speed of 250 rpm. Homogenates were serially diluted with 0.1% sterile Buffered Peptone Water (GranuCult®, Merck, KGaA, Germany). For the detection of *L. monocytogenes*, 0.1 mL of homogenate was spread on PALCAM agar (Code 1440, Fort Richard Laboratories, Auckland) and incubated at 37ºC for 24 - 48 h. Plating was done in triplicate. After incubation, colonies which appeared as a black donut shape with a concaved centre and black halo on PALCAM agar were counted and suspected to be *Listeria* species. The limit of sensitivity for the detection of suspected *Listeria* was 100 colony forming units in 1 g of sample. The DNA of one presumptive *Listeria* colony on each plate was isolated and tested by Polymerase Chain Reaction (PCR).

For *L. monocytogenes* detection with enrichment, a modified version of the ISO 11290-1 method was followed. Twenty-five g of lettuce leaves was homogenised with 225 mL of *Listeria* Enrichment Broth (LEB) (Fort Richard) using a Smasher™ Lab Blender (AES-Chemunix) for 120 s at a speed of 250 rpm. Homogenates were incubated at 30ºC for 24 h. After 24 h, 0.1 mL was transferred into 10 mL Fraser Broth and then incubated at 35ºC for 24 h. 0.1 mL of the solution was streaked onto *Listeria* selective Oxford (Fort Richard) and PALCAM agar (Fort Richard). After incubation at 37 ºC for 24 and 48 h, the DNA of one presumptive *L. monocytogenes* colony on each plate was isolated and tested by PCR. The limit of sensitivity for the detection of *Listeria* in the enrichment procedure was one in 25 g of sample.

3.2.3 Bacteria DNA Isolation and Polymerase Chain Reaction (PCR)

A suspected *L. monocytogenes* colony from each presumptively positive sample was incubated in 9 mL Brain Heart Infusion (BHI) broth (Bacto™ Brain Heart Infusion, Becton, Dickinson Company, Le Pont de Claix, France) for 24 h at 37°C. After incubation, 5 µL of the broth was sub-cultured into 9 mL BHI broth and allowed to grow at 37°C for 24 h. The culture was streaked on PALCAM agar at 37°C for 24 h. A colony was then transferred into 9 mL BHI broth at 37°C for 24 h. Five µL of the suspected *Listeria* culture was used to provide DNA and was transferred into a Platinum Green Hot Start PCR 2X Master Mix (Invitrogen by Thermo Fisher Scientific, Lithuania). The duplex PCR mix consisted of 25 µL of Platinum Green Hot Start PCR 2X Master Mix and 12 µL DNA free water (Invitrogen by life technologies UltraPure™ Distilled water, USA). The primers used for the PCR were: 1 µL of *prs*-F (GCTGAAGAGATTGCGAAAGAAG) and 1 µL of *prs*-R (CAAAGAAACCTTGGATTTGCGG), generic *Listeria* primers as well as 3 µL of *hly* A (CATTAGTGGAAAGATGGAATG) and 3 µL of *hly* B (GTATCCTCCAGAGTGATCGA), specific *L. monocytogenes* primers (Budniak et al., 2016). The PCR master mix with DNA was run with the ProFlex PCR system (Applied Biosystems by life technologies, Singapore). The program of the PCR cycle was: Denaturation at 94°C for 180 s, annealing at 94°C for 24 s, 53°C for 69 s, 72°C for 69 s for 35 cycles, and a final extension at 72°C for 7 min. The amplified PCR products were visualized with agarose gel electrophoresis. The gel electrophoresis was done by pipetting 10 µL aliquots of the amplified PCR product into wells of E-Gel EX Agarose 2%. E-Gel Low Range Quantitive DNA Ladder was used as the marker. *L. monocytogenes* reference strain ATCC 35152 was used as positive control whereas sterile DNA free water was used as a negative control. The agarose gel was then slotted into the iBase of the E-Gel Pre-cast Agarose Electrophoresis System and run for 11 min. A positive test for *Listeria* species

formed a band at 370 bp and a positive result for *L. monocytogenes* amplified at both 370 bp and 730 bp (Budniak et al., 2016).

3.2.4 Statistical analysis

The sample data collected are categorical in nature and as such Chi-Square analysis was used to determine whether bagged lettuce is associated with *Listeria* contamination. The Chi-Square test for independence compares two variables in a contingency table to determine whether the distributions of categorical variables differ from each other. The chi-squared test performs an independency test under the following null and alternative hypotheses, H_0 and H_a, respectively.

H_0: Bagged lettuce is not associated with *Listeria* contamination

H_a: Bagged lettuce is associated with *Listeria* contamination

The test statistic of Chi-Squared test is given as

$$\chi^2 = \sum \frac{(O-E)^2}{E} \; with\; degrees\; of\; freedom\; (r-c)(c-1)$$

Where O and E, respectively represent the observed and expected frequencies, r is the number of rows and c is the number of columns. If the calculated Chi-Square statistic test is greater than the critical value from the Chi-Square distribution table at an alpha level of 0.05, the null hypothesis is rejected, indicating a statistically significant association between bagged lettuce and *Listeria* contamination. The Chi-Squared test applies an approximation assuming the sample is large, however, in cases with small sample size as seen in our study, the Fisher exact test is more appropriate in analysing the data. The analysis in this study was performed with IBM SPSS Statistics for Windows, version 26 (IBM Corp., Armonk, N.Y., USA), which

automatically provides an analytical result of Fisher's exact test as well as Chi-Squared test for 2 x 2 contingency tables.

3.3 Results and Discussion

Table 3.2 shows the results of the survey as analysed with the Chi-Square test. All the lettuce samples that tested positive for *Listeria* were from bagged lettuce. The positive results from the survey are summarized in Table 3.3. *Listeria* was detected in 7 out of 100 lettuce samples surveyed. *L. monocytogenes* was detected in 5 samples while 2 samples were only positive for other *Listeria* species. All the presumptive bacteria DNA amplified at 370-bp representing *Listeria* species (Figure 3.1). Moreover, a unique 730-bp fragment was observed for 2BLL I, 2BLL II, 2BLL IV, BBC III and BBC IV, which is an indication of *L. monocytogenes* (Budniak et al., 2016). Supermarket A had 4 bagged lettuce samples that were positive for *Listeria* with 3 of the positive isolates as *L. monocytogenes*, all from a single product line Brand 2 baby leaf lettuce (2BLL), detected on 3 of the 5 weeks of sampling. Interestingly, *Listeria* was not detected from the baby leaf salad of the same brand (2BLS). Apart from 1BLL III which was only positive with *Listeria* after enrichment, all the other 3 positive lettuce samples from supermarket A (2BLL I, 2BLL II and 2BLL IV) were positive with *Listeria* without enrichment. Supermarket B had 3 bagged lettuce samples that were positive with 2 confirmed to have *L. monocytogenes*. Only one sample from supermarket B (BIL III) was positive for *Listeria* (but not *L. monocytogenes*) without enrichment. *L. monocytogenes* was detected in the 2 samples of baby cos lettuce in 2 successive weeks (BBC III and BBC IV) but only after enrichment.

Table 3.2. Chi-Square test results of *Listeria* contamination in bagged and un-bagged lettuce

Lettuce	Tested Positive for *Listeria*		**Total**
	No	Yes	
Unbagged	40	0	**40**
Bagged	53	7	**60**
Total	**93**	**7**	**100**

$\chi^2 = 5.018, \quad df = 1, \quad p - value = 0.040$

The presence of 130-370 CFU/g *L. monocytogenes* in lettuce without enrichment is a concern for food safety. According to the Australian and New Zealand guidelines for fresh produce safety, *Listeria* should not be detected in 25 g of fresh produce such as lettuce (FPSC, 2019). Salvat & Fravalo (2004) reported that 10 CFU/g of *L. monocytogenes* can cause an unsafe food product in 8 days. The fresh produce processing industry typically sanitize their products before packaging (Olaimat & Holley, 2012). Detection of *L. monocytogenes* might be due to the inefficiency of their sanitizing treatment or contamination of produce in the processing environment before they are put into bags. In addition, the presence of *L. monocytogenes* biofilms on packaging equipment can also be a source of contamination (Galie et al., 2018).

Moreover, damaged bags with holes or perforations have been reported as a medium for pathogen contamination to occur (Williams et al., 2011). None of the samples collected had damaged bags.

Table 3.3. Samples containing *Listeria* in 60 bagged lettuce samples

Supermarkets	**Lettuce sample code**	**Average number (±standard error) of presumptive *Listeria* detected without enrichment (CFU/g)** n=3 technical replicates	**Presence of *Listeria* after enrichment**	**PCR confirmation**
Supermarket A	2BLL I	$3.7 \times 10^2 \pm 0.3$	Positive	*L. monocytogenes* [a]
	2BLL II	$2.7 \times 10^2 \pm 0.3$	Positive	*L. monocytogenes* [a]
	1BLL III	No count	Positive	*Listeria* [b]
	2BLL IV	$1.3 \times 10^2 \pm 0.3$	Positive	*L. monocytogenes* [a]
Supermarket B	BIL III	1.0×10^2	Positive	*Listeria* [a]
	BBC III	No count	Positive	*L. monocytogenes* [b]
	BBC IV	No count	Positive	*L. monocytogenes* [b]

[a] = confirmed in culture from direct plating, [b] = confirmed in culture from enrichment plating

One variety of bagged lettuce in supermarket A (2BLL) tested positive with *L. monocytogenes* contamination for week 1 (2BLL I), week 2 (2BLL II) and week 4 (2BLL IV). Similarly, *L. monocytogenes* was detected in one variety (BBC) from supermarket B on weeks 3 and 4. This suggests that a fresh produce processing factory is having issues with *L. monocytogenes* contamination. It could not establish whether the two contaminated varieties (2BLL and BBC) were from the same supplier. This observation warrants a surveillance study about the prevalence of *L. monocytogenes* in fresh produce processing facilities in New Zealand.

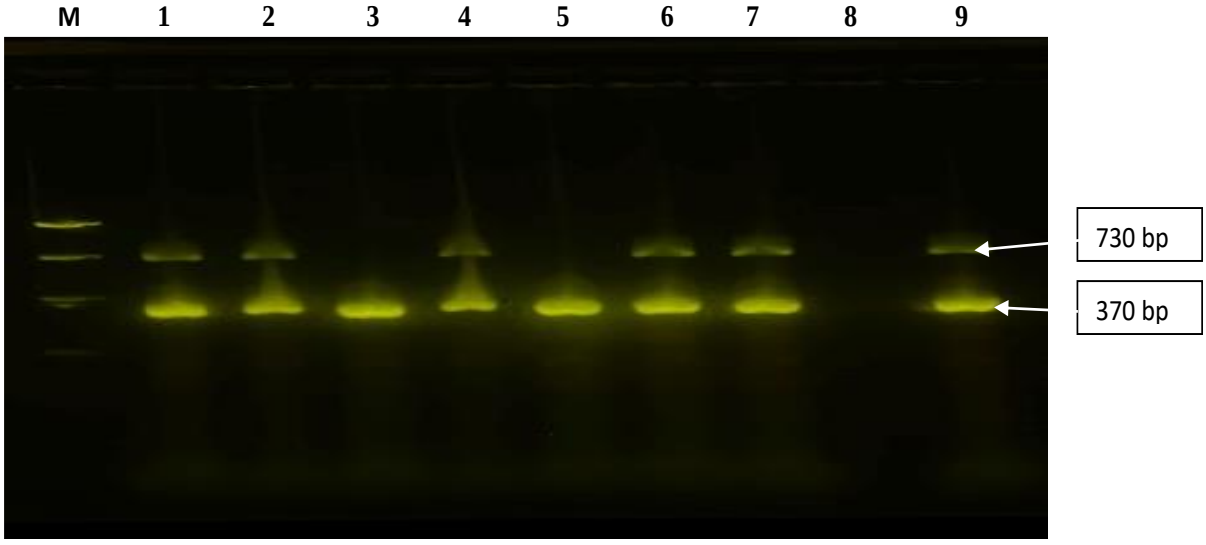

Figure 3.1. Agarose gel electrophoresis showing *Listeria* species and *L. monocytogenes* PCR products. Amplified PCR products of *Listeria* species and *L. monocytogenes* on agarose gel. *Listeria* genus specific *prs* gene amplifies at 370 bp and the *hly* gene of *L. monocytogenes* amplifies at 730 bp. Order of amplified products: M-Ladder, 1-2BLL I, 2-2BLL II, 3-1BLL III, 4-2BLL IV, 5- BIL III, 6-BBC III, 7- BBC IV, 8-Negative Control and 9- *L. monocytogenes* reference strain ATCC 35152 (Positive Control).

Several surveillance studies about the prevalence of *L. monocytogenes* in lettuce sold at retail have been reported. Zhu et al., (2016) conducted a survey about the prevalence of *Listeria* species in 4 different retail shops in Canterbury, New Zealand. They found an average of 4 log CFU/g *Listeria* in bagged lettuce samples in all the retail shops. The results of their work agree

with this results with relatively high counts of *Listeria* present in some lettuces sold in New Zealand retail shops. In another study, ready-to-eat (R.T.E) lettuces packaged by MAP were collected from a factory in Spain and tested for their ability to support *L. monocytogenes* growth. *L. monocytogenes* was able to grow by 4.85 log and 2.66 log CFU/g at 13 and 5 °C respectively after 14 d (Carrasco et al., 2008). This shows that even if only contaminated by low numbers of *L. monocytogenes*, M.A.P-packaged RTE lettuces found in retail shops could be a potential source of foodborne outbreaks if effective produce control practices were not followed. Several other authors have reported high counts of *L. monocytogenes* in salads sold at retail (Little et al., 2007; Sant'Ana et al., 2012) while others did not detect *L. monocytogenes* or reported low counts of *L. monocytogenes* (MPI, 2015, NWMR, 2011). For example, Little et al., (2007) found 2 salad samples contaminated with 1.7×10^2 CFU/g and 9.9×10^2 CFU/g *L. monocytogenes* during a survey of pre-packaged mixed vegetable salads in the UK. In another study, Sant'Ana et al., (2012) found 3.1% of RTE vegetable samples positive with *L. monocytogenes* with counts from 10 to 260 CFU/g. On the other hand, all four salad samples contaminated with *L. monocytogenes* during a survey in North & Western Metropolitan Region (NWMR) in Australia had counts <100 CFU/g. *L. monocytogenes* was not detected in a microbiological survey of leafy salads in New Zealand retail in 2012 (MPI, 2015). Leafy salads used to be in open bins in supermarkets in New Zealand and hence the effect of that environment or any dressing on the salad may have influenced results.

The prevalence of *L. monocytogenes* in RTE lettuce was 3.52 % in Sweden (Althaus et al., 2012). In a similar study conducted in Norway, the prevalence of *L. monocytogenes* in lettuce sold in retail shops was 0.5% (Johannessen et al., 2002). A microbial survey of pre-packaged fresh leafy salads available in retail in New Zealand was conducted in 2012. From a total of 307 salad products, they detected other *Listeria* species (but not *L. monocytogenes*) in 19 samples (6.2 %) (MPI, 2017).

Lettuce processing/sanitization in NZ produce industries varies among the processing industries. A visit to one of the fresh produce industries revealed that the processing steps involve harvesting of produce from the field, washing, cutting, a second triple wash with chlorine-based sanitizers on different platforms and finally packaging. This is the first study which has compared the prevalence of *L. monocytogenes* in bagged and un-bagged lettuces sold in retail shops. We found 5% of *L. monocytogenes* and 2% of other *Listeria* species in our survey in bagged lettuce. A similar survey conducted by (Little et al, 2007) in the United Kingdom found the prevalence of *Listeria* and *L. monocytogenes* on pre-packaged vegetables to be 10% and 4.8% respectively. The high rate of prevalence they found might be due to the different varieties of fresh produce samples they collected whereas this study only focused on lettuce.

Although there are many microbial contamination risks associated with un-bagged salads in supermarkets, *L. monocytogenes* was not detected in any of the un-bagged salad samples. A possible reason might be due to the method used to process our samples before microbial analysis. The outer and discoloured leaves were removed from the un-bagged samples, and this might have affected our results. However, many consumers remove the outer and discoloured leaves from vegetables before consumption. Many consumers might perceive that packaged salads have been processed and sanitized already therefore not washing before consumption. In contrast, this study suggests that bagged lettuces represent a higher risk so consumers should pay more attention to washing them.

My interaction with the staff of the produce section revealed that fresh produce suppliers regularly bring new un-bagged produce and get rid of the un-bagged produce sold on shop floor within 2-3 days. This indicates that, un-bagged fresh produce sold in the supermarket might have a shorter shelf life. However bagged salads can remain on the shop floor until their best

before date is due (which is usually 4-6 days) after packaging. This might give more time for pathogens found on contaminated produce to grow. Most of the bagged lettuce samples were shredded/cut lettuce and this might also contribute to pathogen growth. Likotrafiti et al., (2013) showed that shredded/cut lettuce releases nutrients and water from cut surfaces. The relatively high temperatures (4-7 °C) recorded in the produce section of the supermarkets at the time of collection might also contribute to the growth of *L. monocytogenes*. Due to the psychotrophic nature of *L. monocytogenes* (Rees et al., 2017), it is essential that supermarkets ensure a strict temperature control especially in their produce sections to prevent proliferation of *Listeria*.

An obvious observation, common during sample collection, was the high moisture content of bagged lettuce. Lettuces continue to respire after harvesting as well as when they are packed (Novak, 2010). Respiration rates in fresh-cut lettuce leaves were reported as higher than whole head lettuces due to wounds caused during lettuce processing (Novak, 2010). This can increase the exposed surface area and thereby increasing the respiration rate especially at high temperatures (Manolopoulou et al., 2010; Likotrafiti et al., 2013; Saltveit, 2016). Respiration of lettuce leads to transpiration (the loss of water vapor from surfaces of plants) which consequently increases relative humidity (RH) when they are in bags (Bovi et al., 2016). Future surveys with sampling in all months of the year may give further details about *Listeria* contamination in NZ supermarkets. Studies on the effect of relative humidity in bagged salads are needed to establish the best RH in packages which will minimise pathogen growth while maintaining the quality of salads.

3.4 Conclusion

This survey investigated the occurrence of *L. monocytogenes* in RTE lettuces sold in supermarkets in a major city in New Zealand. 7 % of bagged lettuce samples were contaminated

with *Listeria* while none of the fresh un-bagged lettuce contained *Listeria* suggesting a higher risk of *Listeria* contamination in bagged product compared to whole, un-bagged lettuce. The effect of relative humidity on *Listeria* growth could be a key factor in bagged salads since survival and growth of *L. monocytogenes* is exacerbated under humid conditions. The cutting process used in preparing the lettuce leaves for bagging may also contribute to increased survival and growth by releasing nutrients. An investigation in the ability of lettuce juice to support *L. monocytogenes* growth is needed. The results of this survey should be an alert for the New Zealand Food safety authorities, fresh produce processers, consumers and other stakeholders to ensure strict practices of effective sanitation are applied in the fresh produce supply chain to prevent *Listeria* related outbreaks and recalls. Additional research about novel control methods used in controlling *L. monocytogenes* on lettuces will be important for the produce industry.

3.5 Summary of chapter and the link to the next chapter

The result of this chapter is a clear indication of the need for this PhD research. The prevalence of *L. monocytogenes* in RTE lettuce with relatively high *Listeria* numbers unacceptable for consumption was found in bagged lettuces sold in supermarkets. This can be risky for a community with many immunocompromised individuals with potential for listeriosis infections. The results of this chapter also suggest the inadequacy of the cleaning systems used in the produce industry. Washing lettuces with sterilised water for one minute could also not reduce *L. monocytogenes* numbers below the detection limit. The need for additional research about the extent of *Listeria* attachment to lettuce surfaces under various washing methods will be important for *Listeria* control on produce surfaces. This research work is about comparing *L. monocytogenes* survival on hydroponic and soil grown lettuces to identify the relative risk of these two types of lettuce. It has been hypothesized that hydroponic grown lettuces might

be a better alternative in reducing *L. monocytogenes* survival and spread since they are grown in a controlled environment. Therefore, in the next chapter, a comparison is made between hydroponic and soil grown lettuces to identify the differences in their nutrient content and leaf surface microbiota. For a better understanding of each lettuce type, these differences are important to identify whether they can influence *Listeria* colonisation.

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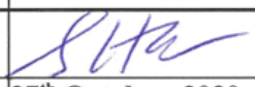
Primary Supervisor's Signature:	
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CHAPTER 4 Differences in nutrients concentration and microbiome community composition between hydroponically and soil grown lettuce leaves

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Abstract

Aims: The use of hydroponics in fresh produce production is a more controlled environment than soil, and therefore may be easier to prevent pathogen contamination. In this study, the differences between hydroponic and soil grown lettuce leaves with respect to the nutrient concentrations as well as the bacterial community composition was analysed.

Methods and Results: Analysis of the major and minor nutrients found in both lettuces revealed the concentrations of sodium, zinc and boron to be higher ($p < 0.05$) in soil grown lettuce than hydroponic grown lettuce. However, there were no significant differences ($p > 0.05$) in the concentrations of nitrogen, phosphorus, potassium, sulphur, calcium and magnesium between soil and hydroponically grown lettuce. 16S rRNA amplicon sequencing revealed that bacteria associated with hydroponically grown lettuce were more diverse than soil grown lettuce. *Pseudomonas* was found to be the dominant genus on hydroponically grown lettuce while *Bacillus* was found to be dominant in soil grown lettuce.

Conclusion: This result is an indication that the use of different growth systems in fresh produce cultivation can affect the nutrient content of the produce as well as the bacterial community composition.

Significance and Impact: Knowledge of the differences in the nutrient content and bacterial composition of hydroponically and soil grown lettuce can be used in the development of fresh produce safety programs especially regarding the ability of leaf microbiota to promote or supress pathogen survival as well as the availability of nutrients which can be used by pathogens.

4.1 Introduction

Foodborne outbreaks associated with fresh produce have been frequently reported (Carstens *et al*., 2019). A recent outbreak of pre-cut melons in several states in the US contaminated with *Salmonella carrau* resulted in 117 cases with 32 hospitalizations (CDC, 2019). A multistate outbreak associated with romaine lettuce contaminated with *E. coli* O157:H7 resulted in 62 cases with 25 hospitalizations (CDC, 2019). *L. monocytogenes* was implicated in a multinational foodborne outbreak associated with frozen vegetables which caused 10 deaths and 54 illnesses (EFSA, 2018). Contamination of fresh produce by pathogens occurs through various means such as cultivation practices, handling and processing (Jung et al., 2014).

The current food control methods used to reduce and prevent pathogenic bacteria in fresh produce have not been able to completely eliminate the risk of contamination with foodborne pathogens (Kyere et al., 2019). In the previous chapter, the prevalence of *Listeria* in bagged lettuces sold in supermarkets of a major city in New Zealand was found to be 7% with relatively high numbers of *Listeria* counts. The incidence of foodborne outbreaks in addition to the findings of other researchers about pathogen control in fresh produce suggest that it is worth improving the current control methods as well as investigating novel food control methods to prevent pathogen contamination of fresh produce. Most of the current control methods involve the use of an external agent such as chemicals for washing, light radiation or an environmental factor applied to the produce (Parish et al., 2003). A more novel approach is encouraging the growth of natural native, non-pathogenic microbiota to competitively exclude pathogens.

The ability of *L. monocytogenes* to survive and grow in a medium or an environment depends on its capability to utilize the nutrients found in that medium or environment (Jarvis et al., 2016). Several studies have described leafy greens as unfavorable media for the survival and growth of *L. monocytogenes* (Jacxsens et al., 1999; Farber et al., 1998) while others have

reported the ability of leafy greens to support *L. monocytogenes* growth (Koseki & Isobe, 2005; Omac et al., 2018). Research to find out whether there are differences in hydroponic and soil grown lettuce leaves nutrients and their potential effect on pathogens survival can be useful in understanding more about fresh produce safety.

The differences between soil and hydroponically grown produce in terms of their nutrient content have been investigated. For example, Treftz et al. (2015) evaluated the differences between the nutrient content (ascorbic acid, α-tocopherol and total phenolics) of hydroponic and soil grown strawberries. They concluded that hydroponic grown strawberries were richer in the selected nutrients than soil grown strawberries. In another study, Selma et al. (2012) reported that hydroponically grown fresh cut lettuce had higher vitamin C and phenolic compounds than soil grown lettuce. Also, the microbiological quality of the hydroponically grown lettuce was higher than soil grown lettuce since lactic acid bacteria and coliform counts for the hydroponic grown lettuce were 3 log and 1.5 log CFU/g lower respectively than soil grown lettuce, which indicates the possibility of differences in nutrients in produce affecting bacterial populations.

In the human microbiota, it has been found that some indigenous species produce antibacterial compounds which reduce the growth of pathogenic bacteria. A study conducted by Zipperer et al. (2016) reported the activity of lugdunin, an antibacterial compound produced by *Staphylococcus lugdunensis* (a commensal which normally thrives in the nasal cavity) against *Staphylococcus aureus* (*S. aureus*) (a pathogen). The bactericidal effect of lugdunin was able to eliminate methicillin-resistant *S. aureus* at 10x the minimal inhibitory concentration (Zipperer et al., 2016). Biocontrol studies on the prevention of pathogenic bacteria on fresh produce using bacteriophages, bacteriocins such as nicin and lactic acid bacteria have also been reported by several researchers (Leverentz et al., 2003; Oladunjoye et al., 2016: Linares-

Morales et al., 2018). However, very little is known about the use of indigenous microorganisms in leafy greens such as lettuce to control pathogenic bacteria.

The use of hydroponic cultivation has been associated with a lower chance for microbial colonisation than soil grown fresh produce since the soil is a big reservoir for diverse microorganisms with some being opportunistic pathogens (Alegbeleye et al., 2018), and therefore comes with a higher risk of contamination. Gomes Neto et al., (2012) investigated the microbiological quality of 180 iceberg lettuce samples from organic (n=60), conventional (n=60) and hydroponic (n=60) farming systems in Brazil and found that hydroponically grown lettuces had the lowest bacteria and parasitic cell counts. *E. coli* O157:H7 was found to have a higher colonisation and internalization potential in soil grown spinach than hydroponically grown spinach (Macarisin et al., 2014).

Several studies have used 16S rRNA phylogenetic marker sequencing (metabarcoding) to determine bacterial composition, diversity and abundance on leaf surfaces (Rastogi et al., 2012; Dees et al., 2014). Dees et al. (2014) used 16S rRNA sequencing to study the bacterial composition of lettuce leaves. They reported that the diversity of the bacterial community found on lettuce leaves 3 weeks after planting was significantly greater than that found at harvest (5 to 7) weeks after planting. In another study, Jackson et al. (2013) found no significant differences in bacterial composition between organic and conventionally grown leafy vegetables. Metabarcoding sequencing of the bacterial microbiome on spinach leaves and rocket salads revealed Proteobacteria to be the predominant phyla followed by Bacteroidetes and Firmicutes (Tatsika et al., 2019). No studies have been done to evaluate the differences in the bacterial composition of soil and hydroponically grown lettuces using 16S rRNA amplicon sequencing. With the previous knowledge about hydroponically grown lettuce available in the

literature, this study can be potentially useful in a biocontrol program in fresh produce safety (Alegbeleye et al., 2018; Macarisin et al., 2014).

In this study, the differences in the nutrient content as well as the bacterial composition of hydroponically and soil grown lettuce were investigated. It was hypothesized that the different growth systems used for lettuce cultivation would influence the nutrients and bacterial community associated with lettuce leaves. This knowledge is important to understand the dominant bacterial phyla on lettuce grown under different conditions and whether the bacterial community can have the ability to promote or suppress the survival of pathogenic bacteria on fresh produce.

4.2 Materials and Methods

4.2.1 Plant material

Buttercrunch lettuce seeds (*Lactuca sativa* L. var. *capitata*) were originally sown and maintained on soil potting mix (pH 6.4-6.5) or in hydroponic solution. Both soil and hydroponic lettuces were grown in the greenhouse of the Plant Growth Unit, Massey University, New Zealand from October to December 2018. The temperature of the greenhouse was maintained at 20 °C. The soil grown lettuces were cultivated in thin rows, 35 cm apart and a depth of 5 mm (Sanguandeekul, 1999). The soil potting mix used is from a commercial source (Daltons base mix) with its components provided in Table 4.2. The same source of water was used to water lettuces grown in soil potting mix and in the hydroponic solution. The hydroponic solution was maintained at an Electrical Conductivity (EC) between 1.2 to 1.3 dS/m and the pH was controlled daily at 5.8. Lettuce plants were used for experiments four weeks after planting. The same water source was used to water lettuces in potting mix as well as the hydroponic nutrient solution.

4.2.2 Preparation of lettuce samples for nutrients analysis

Nutrient analysis was done by Hills Laboratories, Hamilton, New Zealand. 18 sets of lettuce leaves from each of hydroponically and soil grown lettuce (grown under the conditions described above) with each weighing 5 g were aseptically transferred to the laboratory. Samples were oven-dried at 62°C overnight and ground to pass through a 1.0 mm screen. Analytical results were reported from this sample fraction and were corrected for residual moisture (typically 5%), unless units denoted as percentage dry matter (% DM). For the nutrient analysis, their concentrations were presented as % DM for the major nutrients and mg/kg DM for the minor nutrients. Nitrogen analysis was done by Dumas combustion. The remaining nutrients were analysed for by nitric acid/hydrogen peroxide digestion followed by inductively coupled plasma-optical emission spectrometry (ICP-OES). This same procedure was repeated three times with different sets of leaves from a single harvest with the growth conditions described above. Table 4.1 shows the components of the hydroponic stock solutions used to grow lettuce for this study. Table 4.2 shows detailed information for the soil potting mix used for growing lettuce used in this study.

Table 4.1. Components of hydroponic stock solution used for growing lettuce

Feed Nutrients	Amount in grams per litre (g/L)
A solution	
Calcium nitrate	105
Potassium nitrate	54
Iron chelate-EDTA	5
B solution	
Mono ammonium phosphate	8.7
Potassium nitrate	79
Magnesium sulphate	58
Mono potassium phosphate	16.3
Manganous sulphate	0.2
Zinc sulphate	0.2
Copper sulphate	0.035
Boric Acid	0.35
Ammonium molybdate	0.01

Table 4.2. Components of soil potting mix used for growing lettuce

Potting mix nutrients	Amount
Dolomite	150 g
Short term fertilizer	150 g
Total Nitrogen (N)	14%
Ammoniacal Nitrogen	2.8%
Water Insoluble Nitrogen	5.8%
Urea Nitrogen	2.7%
Other water soluble Nitrogen	2.7%
Phosphorus (P)	6.0%
Potassium (K)	11.6%
Magnesium (Mg)	1.0%
Sulphur (S)	4.0%
Iron (Fe)	1.0%
Manganese (Mn)	0.5%

4.3 Processing of lettuce for DNA extraction

Hydroponic and soil grown lettuces were collected on a weekly basis for DNA isolation. Leaves from individual plants were thoroughly mixed to ensure representative subsampling. Both hydroponic and soil leaves were randomly collected to a total weight of 100 g. Leaves were aseptically transported to the laboratory and washed with running distilled water to remove any dirt and loose contaminants. Lettuce leaf processing was done as described by Jackson *et al*. (2013). Lettuce leaves were homogenized with a Kenwood Processor FDM785BA, Germany, for 3 min. After homogenization, 50 ml of the homogenate was passed through a sterile 11 Whatman 1 filter (11 µm pore size) to remove residual leaf particles. The filtrate (30 mL) was centrifuged for 4400 x g for 20 min at room temperature (Eppendorf Centrifuge 5702, Hamburg, Germany) and DNA was isolated from the resulting pellet. DNA was extracted using the Nucleospin® Tissue genomic DNA kit (Machery-Nagel GmbH, Germany) according to the manufacturer's instructions. The concentration and quality of extracted DNA was checked with the Colibri Microvolume Spectrometer (Berthold Detection Systems GmbH, Germany) and was also run on 1.5% agarose gel. DNA was isolated from seven hydroponic grown lettuce and 11 soil grown lettuce. In total, 18 DNA samples were obtained.

4.4 Microbial 16S rRNA sequencing

DNA samples were sent to New Zealand Genomics Ltd (NZGL: Massey Genome Service at Massey University, Palmerston North) to be sequenced using the Illumnina MiSeq Sequencing Platform. A 16S rRNA library for each of the 18 samples was prepared using PCR to amplify the V3-V4 hypervariable regions. The forward primer that was used was 16SF (5'-CCTACGGGAGGCAGCAG-3') and the reverse primer was 16Sr (5'-

GGACTACHVGGGTWTCTAAT-3') (Kozich et al., 2013). The PCR program consisted of an initial 95°C denaturation for 2 min, a 30-cycling program of 95°C for 20 s, 55°C for 15 s, 72°C for 5 min, and a final elongation step at 72°C for 10 min. Sterile distilled water was used as the negative control. PCR products were purified with magnetic bead capture and amplicons were sequenced on Illumina MiSeq as 2x 250 bp paired-end runs.

4.5 Data analysis

For the nutrient analysis, the amount of major nutrients was recorded in % dry matter whilst the amount of minor nutrients was recorded in mg/kg. Experiments for nutrient analysis was done with 3 biological repeats. The results were expressed as mean ± SD (error bars). Analysis of Variance (ANOVA) and Tukey's test were performed at 95 % ($p<0.05$) confidence level using Minitab Statistical Software (Minitab version 17, State College, Pennsylvania, USA). The sequence reads from the 18 16S rRNA amplicons were analysed with QIIME (version 2018.2) (Bolyen et al, 2019). Steps in this pipeline included pair-joining, denoising, chimera checking and removal, and clustering into operational taxonomic units (OTUs) through dereplication. This processing was performed using DADA2 (Callahan et al., 2016). All samples were then rarefied to the level of the smallest sample size (25588) and any possible loss of information was ruled out by examination of the rarefaction plots. The taxonomic classification of each OTU was determined using the embedded Naïve Bayes fitted classifier, trained on the Silva v. 123 99% identity database. Samples dominated with chloroplast reads were excluded from the analysis.

Alpha and beta diversity analyses were performed to assess within- sample and between-sample diversity respectively. Alpha diversity metrics included the number of observed OTUs, evenness, the Shannon index, and Faith's PD. Significant differences between sample groups

for alpha diversity were tested using the rank-based Kruskal-Wallis one-way analysis of variance. Beta diversity metrics included the Jaccard index, Bray-Curtis dissimilarity, and weighted and unweighted UniFrac distance (Cox et al., 2010) and significance was tested using pairwise PERMANOVA. Further taxonomic comparisons were performed using MEGAN (Huson et al., 2016) with SplitTree functionality (Huson et al., 2016). Analysis of Composition of Microbiomes (ANCOM) was used to detect significant differences in genus-level abundances between sample groups (Mandal et al., 2015).

4.5.1 Availability of supporting data

All raw sequences used in this study are available in the NCBI Sequence Read Archive under study accession number PRJNA599485.

4.6 Results and Discussion

4.6.1 Nutrient concentration of lettuce leaves

The concentrations of sodium, zinc and boron were significantly higher ($p < 0.05$) in the soil grown lettuce leaves than the hydroponic lettuce leaves. The other nutrients (iron, manganese**,** copper, nitrogen, phosphorus, potassium, sulphur, calcium and magnesium) did not show significant differences ($p > 0.05$) between the soil and hydroponic grown lettuce leaves (Figures 4.1 and 4.2).

The higher concentrations of the sodium, zinc and boron in the soil grown leaves may be due to the relatively higher pH (6.4-6.5) of the soil potting mix as compared to the hydroponic solution pH (5.8). The adsorption of boron by plants depends on the pH of the growth medium (Bingham et al., 1971). Higher pH values result in higher adsorption of nutrients by plants. Boron uptake by plants growing in soil increased when the pH was increased from 3 to 8

(Bingham et al., 1971). However, Goldberg and Glaubig (1986), found that the adsorption of boron decreased when the pH was increased to a range of 10 to 11.5. Iron, copper, zinc, boron, and manganese have been reported to become inaccessible to plants at pH values above 6.5 (Timmons et al., 2002; Tyson, 2007). Similar results have been reported for sodium (Aboukarima et al., 2018) and zinc (Jahiruddin et al., 2008) where optimum pH values resulted in high adsorption rates. The studies indicate that, optimum pH values for a growth medium are essential for the adsorption of trace elements from the growth medium to plants.

The wide variations between the different nutrients of both soil and hydroponic grown lettuce such as approximately 200 mg/kg for manganese and approximately 30-40 mg/kg for boron, fall in the range of what others (Hartz & Johnstone, 2007; Hochmuth et al., 1991; Jones et al., 1991) have reported. In this study, copper was found to be the lowest micronutrient (Fig. 4.2) and this agrees with what other researchers have found (Hochmuth et al., 1991; Jones et al., 1991). Hartz & Johnstone (2007) reported that the relative concentrations of different nutrients in leaves were very similar among different growth stages (early heading and pre-harvest) and different locations.

The results from this study indicate partial differences in some trace elements between hydroponically grown and soil grown lettuce leaves. Further studies to determine whether these differences will affect *L. monocytogenes* survival on lettuce surfaces and in lettuce juice will be important to be carried out.

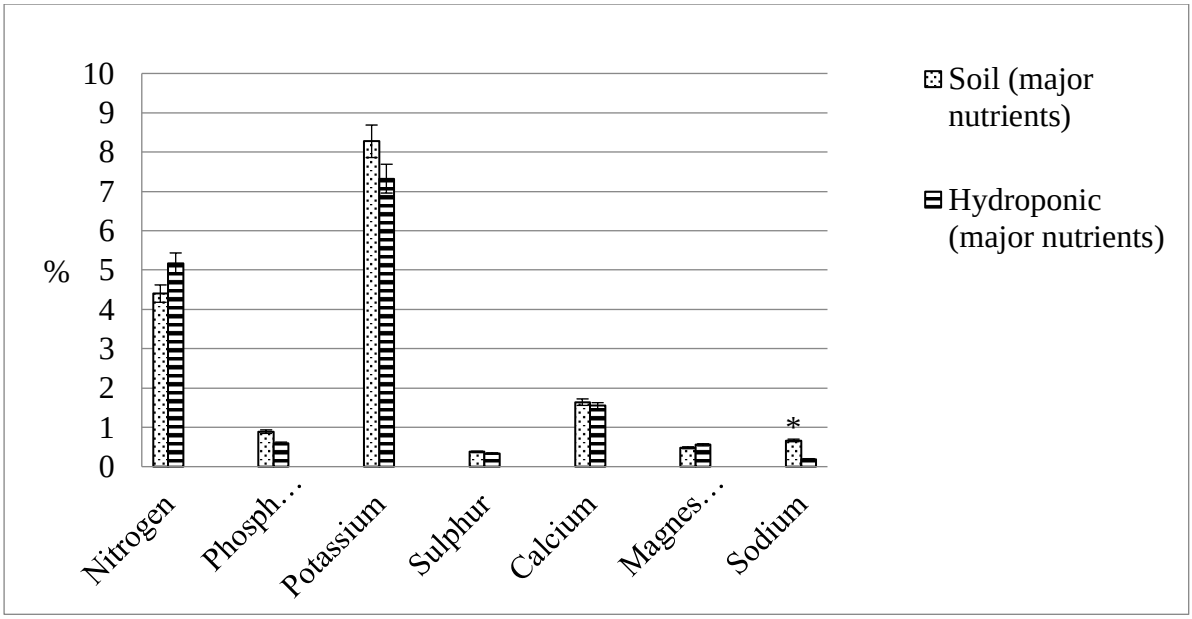


Figure 4.1. Comparison of major chemical elements (% dry matter) found in soil and hydroponically grown lettuce leaves. Comparison of major chemical elements (% dry matter) found in soil and hydroponically grown lettuce leaves represented by the mean of three biological repeats with standard deviation error bars. Statistical differences were performed with ANOVA and Tukey's test at $p < 0.05$. Bars denoted with an asterisk (*) indicate statistical significance.

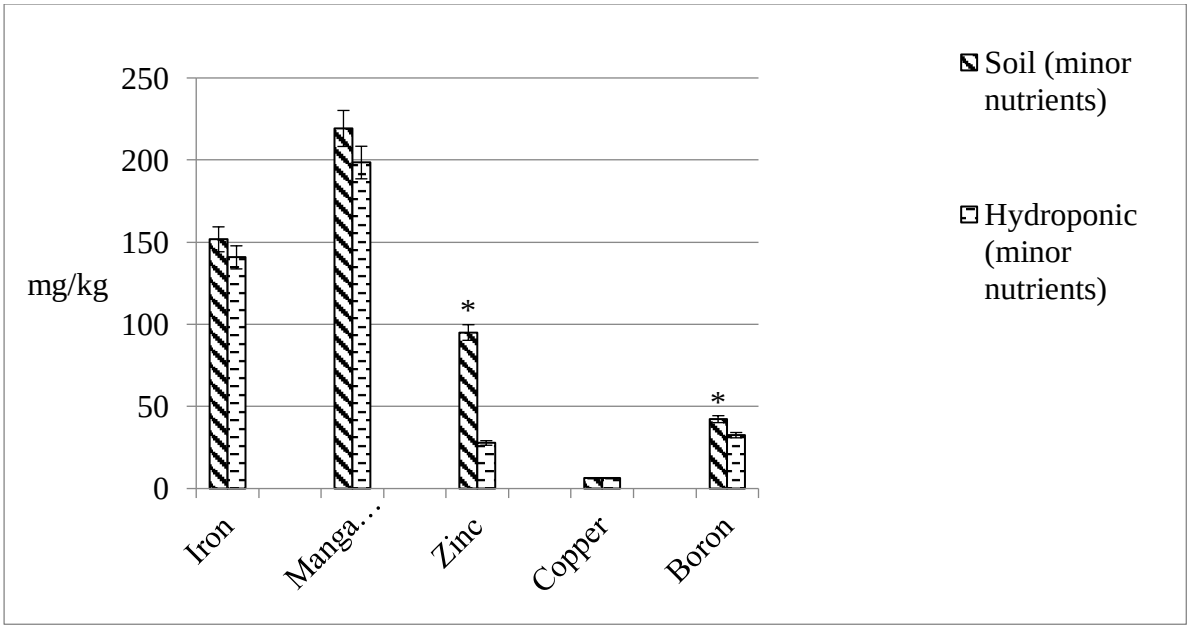


Figure 4.2. Comparison of minor chemical elements (mg/kg) found in soil and hydroponically grown lettuce leaves. Comparison of minor chemical elements (mg/kg) found in soil and hydroponically grown lettuce leaves represented by the mean of three biological repeats with standard deviation error bars. Statistical differences were performed with ANOVA and Tukey's test at $p < 0.05$. Bars denoted with an asterisk (*) indicates statistical significance.

4.6.2 Microbial sequencing with Illumina MiSeq

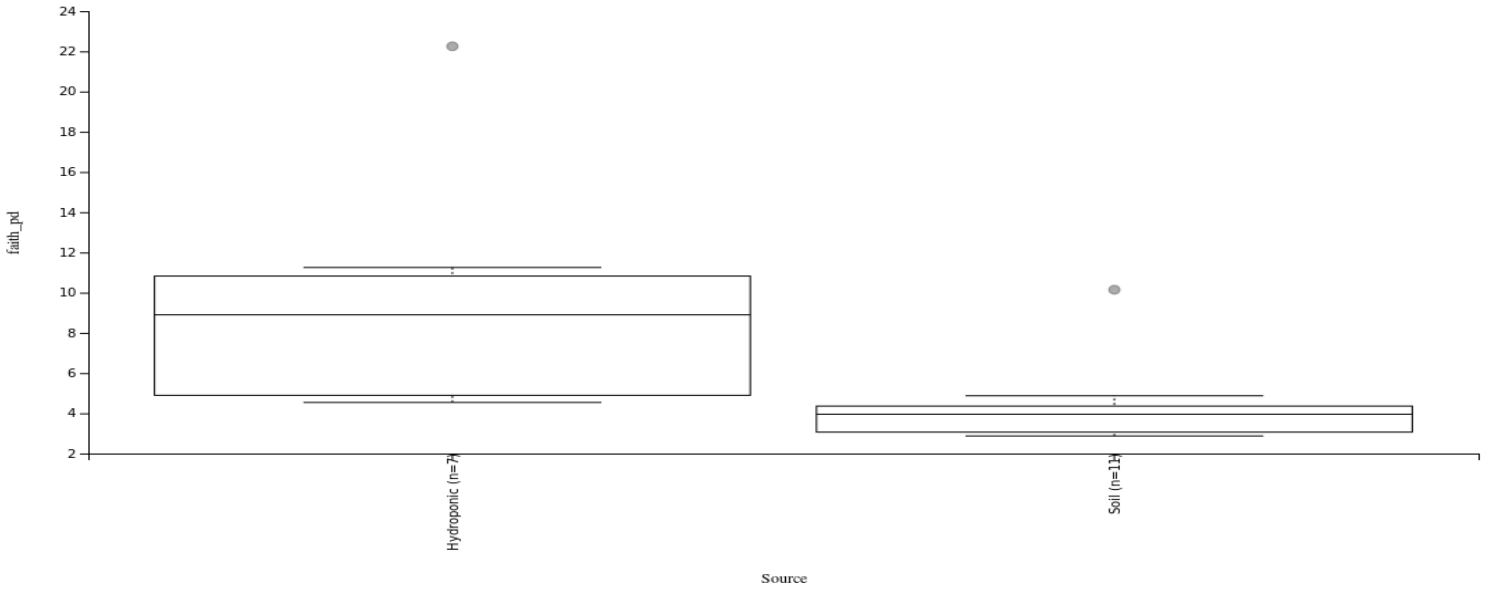
In this study, 16S rRNA amplicon sequencing was used to characterize the bacterial community associated with soil and hydroponically grown lettuce leaves. Overall, 832550 reads were recovered from the 18 lettuce samples used. We were able to rarify all the samples at the threshold given by the next least abundant sample MGS00145-6 (Table 4.3).

Alpha diversity was quantified by several diversity indices, including Faith's PD, Shannon and the number of observed OTUs and evenness. Statistical testing by all indices showed no difference in hydroponic and soil grown lettuces for the Shannon index. On the other hand, there was a significant difference ($p < 0.05$) observed between hydroponic and soil grown lettuce when quantified by Faith's PD. This demonstrates that the bacterial community on hydroponic grown lettuce is more phylogenetically diverse than the bacterial community found on soil grown lettuce (Fig. 4.3). This strong signal from Faith's PD suggests that the differences in internal phylogenetic diversity between the soil and hydroponically grown lettuce should be further investigated.

Beta diversity was quantified using the Jaccard index, Bray-Curtis dissimilarity as well as the weighted and unweighted UniFrac distance metrics. All the metrics used indicated that the bacterial communities identified in the soil grown lettuce were significantly different ($p < 0.05$) from those identified in the hydroponic grown lettuce. This can be seen in the MEGAN phylogram generated from the data (Fig. 4.4). The majority of the bacteria identified from hydroponic grown lettuce (blue rings) were more closely clustered together MGS00145-1 to MGS00145-4 and MGS00145-5 to MGS00145-8) whereas the bacteria identified from the soil grown lettuce (orange rings) also clustered together (Fig. 4.4).

Table 4.3. Total read numbers and filtered read numbers before rarefaction

Sample ID	Paired reads	Filtered reads	Source
MGS00145-1	32697	29672	Hydroponic
MGS00145-2	38901	35708	Hydroponic
MGS00145-3	33804	26563	Hydroponic
MGS00145-4	40427	36261	Hydroponic
MGS00145-5	65331	60298	Hydroponic
MGS00145-6	29173	25588	Hydroponic
MGS00145-8	54314	45958	Hydroponic
MGS00145-14	53444	50171	Soil
MGS00145-15	45000	42544	Soil
MGS00145-16	38109	35618	Soil
MGS00145-17	65539	58338	Soil
MGS00145-18	36648	34558	Soil
MGS00145-19	51024	47716	Soil
MGS00145-20	54831	51395	Soil
MGS00145-21	41571	39005	Soil
MGS00145-23	54778	51296	Soil
MGS00145-24	45226	42205	Soil
MGS00145-25	51733	44203	Soil
Total reads	832550		



Kruskal-Wallis (all groups)

H: 8.663704

p-value: 0.003246

q-value: 0.003246

Figure 4.3. Differences in alpha diversity between soil-grown and hydroponic lettuce. Faith's phylogenetic diversity metric was used to identify significant differences in alpha diversity between our two sample types. Hydroponic samples (left) differ significantly ($p < 0.05$, Kruskal-Wallis test) from soil-grown samples (right), with the bacterial community on hydroponic lettuce more diverse than the bacterial community on soil-grown lettuce.

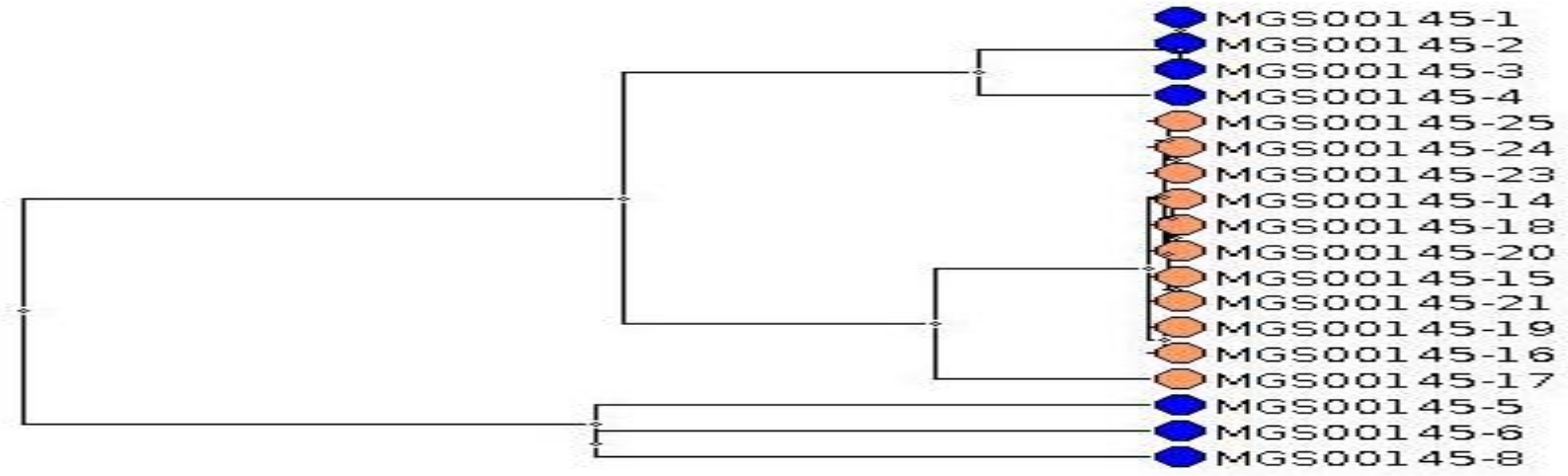


Figure 4.4. Bacterial community composition clusters by growth conditions. MEGAN was used to generate a phylogram of community composition at the genus level. Blue circles indicate hydroponic-grown lettuce samples, while brown circles indicate soil-grown lettuce samples. Samples from the same growth conditions tend to cluster together, indicating that bacterial communities are distinct in soil-grown vs hydroponic lettuce.

To determine which bacterial groups are driving the observed differences in beta diversity between soil-grown and hydroponic lettuce, the abundances of specific taxa in the dataset was investigated. Firmicutes was the dominant phylum in soil grown lettuce samples, while Proteobacteria were dominant in 85% of the hydroponic grown lettuce samples (Fig. 4.5). At the genus level, the dominant bacteria found in the soil grown lettuce were *Bacillus* spp. (54.8% of total reads) whereas *Pseudomonas* were the dominant in hydroponically grown lettuce (16.3% of total reads). Out of the seven hydroponic lettuce samples, four of them (MGS00145-1, MGS00145-2, MGS00145-3 and MGS00145-4) had on average 80% of their amplicons assigned to *Pseudomonas* (Fig. 4.5). *Staphylococcus* (5.4%) *Stenotrophomonas* (3%) and *Rhizobium* (7.2%) are the other genera that were found in the hydroponically grown lettuce. The soil grown lettuce DNA samples were dominated by *Bacillus* (Fig. 4.5).

Further investigations about the differences in community composition between sample groups by examining the abundances of bacterial families was conducted. Although, *Bacillaceae* and *Pseudomonadaceae* were dominant community members on soil-grown and hydroponic lettuce respectively, members of these families were detected in both sample groups. Some families, including *Bacterodaceae, Bifidobacteriaceae, Enterobacteriaceae, Carnobacteriaceae, Streptococcaceae* and *Lachnospiraceae* were observed at low to medium abundance (0.15%-3.15% of total reads) in most samples from both soil and hydroponically grown lettuce. Others were preferentially detected in one group or the other, despite relatively low abundances; these included *Fusobacteriacaea* (hydroponic), *Acidaminococcaceae* (hydroponic), *Microbacteriaceae* (soil grown) and *Veillonellaceae* families (hydroponic). These results highlight subtle differences between community composition of the two tested lettuce types.

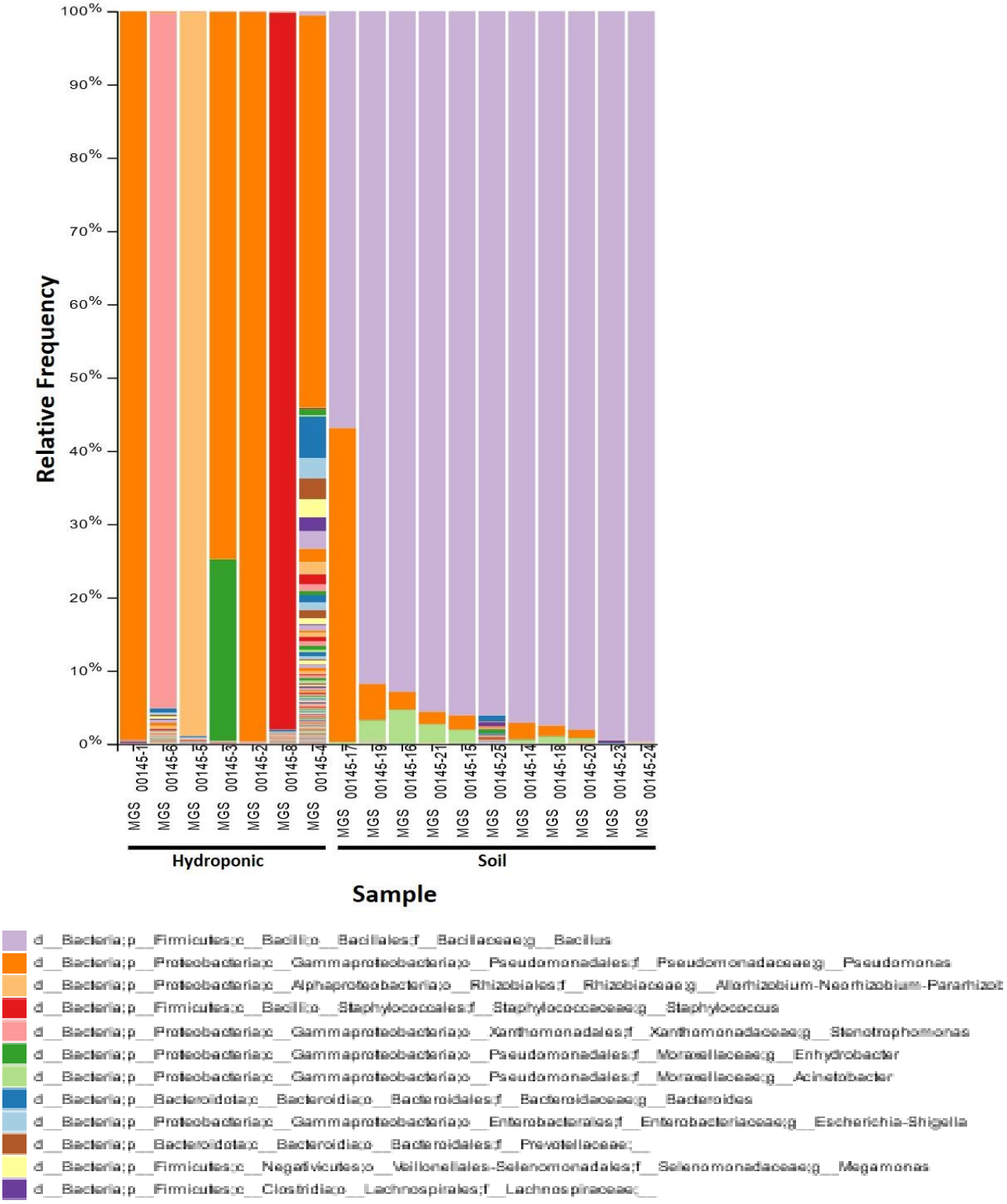


Figure 4.5. Genus level community composition. To further investigate the underlying differences in bacterial community composition between hydroponic and soil-grown lettuce, analysis of the proportion of read assigned to each sample at the genus level was conducted. Members of Firmicutes, in particular *Bacillus*, were dominant in soil-grown lettuce communities, while members of Proteobacteria were dominant in hydroponic lettuce communities, with many of these groups classified as *Pseudomonas*.

An ANCOM statistical test was used to calculate which bacterial genera and species differ significantly between the soil and hydroponically grown lettuce. The ANCOM analysis identified two bacterial genera, *Bacillus* and *Prevotella* with a significant W value (Table 4.4). Two species, *Bacterioides plebeius* and *Bacterioides coprocola* showed markedly high W value compared to the other species tested. These differences at both taxonomic levels further support the differences between soil-grown and hydroponic grown lettuce identified via beta diversity metrics.

Table 4.4. Species with significantly different abundances on hydroponic vs soil grown lettuce

Species	**Reject null hypothesis**	**W**
d__*Bacillus;* __	True	282
d__*Prevotella*; __	True	254
d__Bacteroides_plebeius	False	251
d__*Bacteroides_coprocola*	False	195

4.6.3 Discussion of microbial sequencing with Illumina MiSeq

This study is the first study to analyze the differences between the bacterial communities associated with hydroponic and soil grown lettuce using 16S rRNA amplicon sequencing. *Firmicutes* were identified as the most abundant phylum in the soil grown lettuce microbial community and *Proteobacteria* as the most abundant in hydroponic lettuce communities (Fig. 4.5). Other researchers have also identified these phyla in lettuces (Dees et al., 2014; Jackson et al., 2013). These findings revealed that bacteria associated with hydroponically grown lettuce were more phylogenetically diverse than soil grown lettuce (Fig. 4.3). The increase in microbial diversity in plants and other higher organisms is positively correlated with plant health (e.g. increase in resistance to pathogen colonisation) (Van Elsas et al., 2012; Yan et al.,

2017). Yan et al. (2017) identified that a higher microbial diversity in the rhizosphere of the ragwort plant (*Jacobaea vulgaris*) was found to be associated with higher functional traits. Similarly, a study by Van Elsas et al., (2012) found that a decline in the bacterial diversity in soil led to a better survival and growth of *E. coli* O157:H7. A higher bacteria diversity of lettuce was able to reduce the colonisation of lettuce by *Salmonella enterica* (Klerks et al., 2007). The effect of a high microbial diversity on pathogen colonisation might be due to the presence of space utilized by these diverse groups of bacteria as well competition for available nutrients (Garbeva et al., 2004). Such competition may prevent the proliferation of a newly arrived foreign pathogen.

The dominant bacterial genus on hydroponically grown lettuce was *Pseudomonas*. *Pseudomonas* has been found to be able to compete and reduce the biofilm formation of foodborne pathogens (Alavi & Hansen, 2013; Norwood & Gilmour, 2001). Co-culturing of *L. monocytogenes* with *Pseudomonas fragi* reduced the biofilm forming ability of *L. monocytogenes* when compared with the biofilms formed by a monoculture of *L. monocytogenes* (Norwood and Gilmour, 2001). Similarly, *Pseudomonas fluorescens* (*P. fluorescens*) reduced the biofilm formation of *L. monocytogenes* in a dual species biofilm on stainless steel (Alavi & Hansen, 2013). On the other hand, the ability of *Pseudomonas* to increase the survival of foodborne pathogens has been reported. A surface colonized by *Pseudomonas putida* (*P. putida*) was able to increase the attachment of *L. monocytogenes* (Hassan et al., 2004). A mixed biofilm of *L. monocytogenes* and *P. fluorescens* that was studied with confocal microscope revealed *L. monocytogenes* biofilms located at the bottom of *Pseudomonas* biofilms on glass coverslips (Puga et al., 2014). Ibusquiza et al., (2012) reported that the presence of *P. putida* increased the resistance of *L. monocytogenes* biofilm formation to benzalkonium chloride (a disinfectant). The examples above suggest that a dual biofilm of *L. monocytogenes* and some *Pseudomonas* strains can be a concern for food safety since surface

washing might not be able to remove *L. monocytogenes* biofilms and also some *Pseudomonas* strains can enhance the biofilm formation of *L. monocytogenes* (Puga et al., 2014; Ibusquiza et al., 2012).

The bacterial groups identified on hydroponic grown lettuce have also been reported by other investigators on leafy greens. Dees et al. (2014) observed *Pseudomonas* from leafy green salads in an experiment conducted to find out the effect of different seasons on bacterial community composition. *Stenotrophomonas* was observed on conventionally grown salads using 16S rRNA gene amplicon sequencing (Jackson et al., 2013). *Staphylococcus* (Jackson *et al*., 2013) as well as *Rhizobium* (Soderqvist et al., 2017) have been identified as part of the lettuce leaf microbiota. The controlled environment in a hydroponic set up where nutrient supply, pH, temperature and electrical conductivity are monitored leads to proper growth and development of the plant (Kyere et al., 2019). Such ideal conditions may also favor the growth of many bacteria which might possibly be the reason for a higher microbial diversity (Berg et al., 2017).

Bacillus was determined as the most dominant bacteria on soil grown lettuce. Other researchers have also reported the presence of *Bacillus* on lettuce (Rastogi et al., 2012). Soil is known to contain extraordinarily high levels of microbial diversity (Maron et al., 2018). However, this results indicated lower diversity of bacteria in soil grown lettuce than on hydroponic lettuce. The ability of some bacteria found in soil to produce antagonistic effects on other bacteria might be the reason for the low bacteria diversity we found in soil grown lettuce. *Bacillus subtilis* isolated from soil was found to inhibit the growth of a soil-borne *E. coli* isolate (Sheikh, 2010). This was possibly due to the presence of subtilin, an antimicrobial compound produced by *B. subtillis* which has been found to show antimicrobial effects on bacteria such as *E. coli* and *S. aureus* (Aslim & Beyatli, 2002; Prabhakar & Prabhakar, 2008).

It is worth mentioning that, even though internalization of bacteria from the roots to the upper parts of the plant is possible, not all bacteria have this potential to internalize (Wright et al., 2017). The interaction between *Bacillus* and other pathogenic bacteria on fresh produce has been reported. An increase in the abundance of *Bacillus* was found to have a positive correlation in the number of *L. monocytogenes* on spinach (Soderqvist et al., 2017). The identification of *Bacillus* on both soil and hydroponically grown lettuce is consistent with other studies that have found similar bacteria communities on certain produce types such as lettuce and spinach (Yu et al., 2018; Leff and Fierer, 2013).

This study found differences in the microbiomes of soil and hydroponically grown lettuces. The results are a clear indication that the use of different growth systems in fresh produce cultivation can shape the bacterial community of the produce as was speculated by Dees et al. (2014). Leaf surface properties and levels of nutrients found on leaves have also been found to influence the lettuce leaf microbiome (Hunter et al., 2010). On the other hand, Tatsika et al. (2019) investigated the effect of household washing treatments on the bacterial community prevalent on rocket salad at harvest and before consumption. They found out that household washing treatments did not change the microbial diversity, which was consistent between harvesting and before consumption. This suggests that any interventions in the microbiota of lettuce must occur during growth, rather than relying on post-harvest washing. The results of this work suggest that hydroponically grown lettuce may have a higher colonisation resistance to pathogens due to its higher bacterial diversity compared with soil-grown lettuce. The bacterial taxa endemic to both hydroponic and soil-grown lettuce was also identified, indicating changes in bacterial community composition due to differing growth systems. Further research into the microbiota of hydroponic lettuce should investigate the impact of these differences on the incidence of food-borne pathogens.

4.1 Conclusion

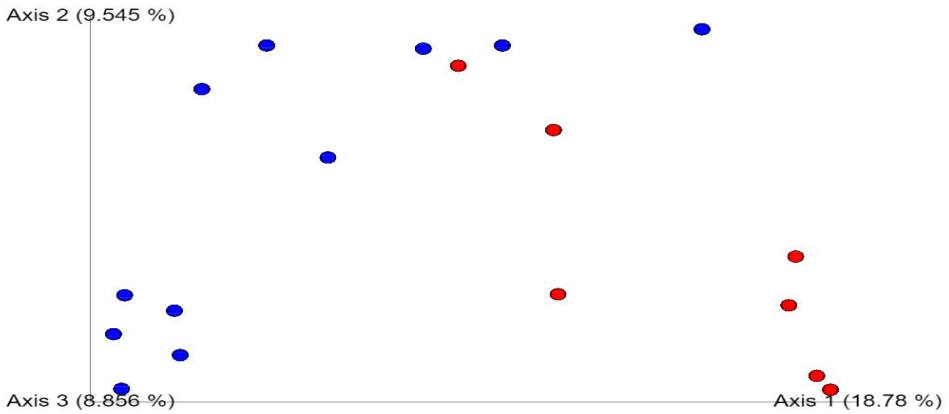
The results of this work demonstrate that different growth systems (hydroponic and soil) used in lettuce cultivation affect the nutrients found in lettuce and microbiota associated with lettuce. Differences in the different lettuce type microbiota have been found to have the potential of affecting the colonisation of fresh produce by pathogens. Future work to compare how *L. monocytogenes* attaches and forms biofilms on both hydroponic and soil grown lettuces is important to be investigated. This will enable us to see whether the differences observed in nutrients and microbial composition might affect the colonisation of lettuce by *L. monocytogenes*.

4.2 Summary of chapter and the link to the next chapter

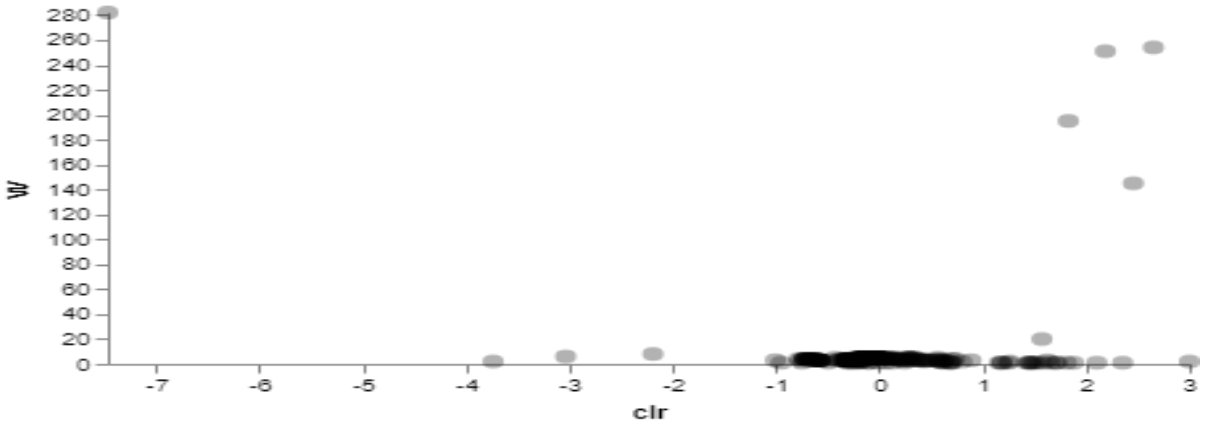
The results of this chapter have revealed the differences between hydroponic and soil grown lettuce leaves in terms of the nutrients as well as the leaf surface microbiota. There were significant differences ($p < 0.05$) in the concentrations of sodium, zinc and boron between hydroponic grown and soil grown lettuces. Moreover, significant differences ($p < 0.05$) were found in the microbiomes of soil and hydroponically grown lettuces. The differences found between the two lettuce types especially with regards to the indigenous microbiota suggest that hydroponically grown lettuce may have a higher colonisation resistance to pathogens due to its higher bacterial diversity compared to soil-grown lettuce. Therefore, in the next chapter (chapter 5), the ability of *L. monocytogenes* to attach, survive and grow on hydroponic and soil grown lettuce (grown under the same conditions in chapter 4) will be investigated. The three *L. monocytogenes* strains (O8A06, O8A07 and O8A08) which will be used are relevant to the study since they were isolated in New Zealand from coleslaw and cabbage. These strains were used because they have been subtyped and their genomic sequences are also known. The results

will enable us to know whether the differences found in the current chapter (chapter 4) will affect the survival and growth of *L. monocytogenes*.

4.1 Supplementary information



Supplementary information S4.1. Differences in beta diversity between soil grown and hydroponic grown lettuce using Jaccard index. Blue circles indicate soil grown lettuce while red circles indicate hydroponic grown lettuce.



Supplementary information S4.2. An ANCOM collapsed species source volcanic plot showing bacterial genus and species (*Bacillus*, *Prevotella*, *Bacteroides plebeius* and *Bacteroides coprocola*) with high W values.

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Name of candidate:	**Emmanuel Owusu Kyere**
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In which chapter is the manuscript /published work: **Chapter 4**	

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CHAPTER 5 Attachment, survival and growth of *Listeria monocytogenes* to hydroponic and soil grown lettuce leaves

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Abstract

L. monocytogenes has been implicated in many foodborne outbreaks involving fresh produce such as lettuce. In order to contaminate the lettuce, the pathogen must attach to the fresh produce. In this study, the attachment under different exposure times (1 s, 10 s, 30 s, 60 s, 2 min and 5 min), survival and growth of *L. monocytogenes* strains O8A06, O8A07 and O8A08 to hydroponically and soil grown lettuce was investigated. Attachment of *L. monocytogenes* O8A08 to hydroponically grown lettuce leaves during 2 and 5 min exposure times was significantly higher ($p < 0.05$) than O8A06 and O8A07. There was no significant difference ($p > 0.05$) in *Listeria* attachment to both hydroponic and soil grown lettuce leaves as well as the number attaching to lettuce between the different exposure times tested. The number of *L. monocytogenes* which attached to both soil and hydroponically grown lettuce leaves within these short exposure times for all the three strains ranged from 0.77 to 1.46 log CFU/cm^2. There was no significant difference ($p > 0.05$) in *L. monocytogenes* survival on both hydroponic and soil grown lettuce. These findings suggest that both lettuce types support the growth of *L. monocytogenes* and any exposure of lettuce leaves to a source of *L. monocytogenes* may result in rapid colonisation of the product. Prevention of fresh produce contamination by *L. monocytogenes* is more important than depending on other control systems to remove contamination.

5.1 Introduction

L. monocytogenes is a Gram-positive bacterium which has been implicated in many fresh produce related outbreaks and recalls (Magalhaes et al., 2014). Ingestion of foods contaminated with *L. monocytogenes* can lead to listeriosis, a disease caused by *Listeria's* invasion of sites along the gastrointestinal tracts especially in immunocompromised individuals and pregnant women (McLauchlin, 1997). In its severe form, it can cause abortion, sepsis, and meningoencephalitis (McLauchlin, 1997).

L. monocytogenes contamination of packaged salads brought about a multistate outbreak in the United States causing 19 hospitalisations and one death (CDC, 2016a). In a similar multistate outbreak in the United States, three people died and nine people were hospitalised due to *L. monocytogenes* contamination of frozen vegetables (CDC, 2016b). The recent listeriosis outbreak in 2018 involving Australian cantaloupes which caused five deaths and many illnesses was due to *Listeria* found on the packing floor of the factory (NSW Food Authority, 2018). The second deadliest outbreak of foodborne listeriosis in the United States occurred in 2011 where cantaloupes and packing equipment in a farm were contaminated with *L. monocytogenes* resulting in 147 people infected with 33 deaths (CDC, 2012).

The first point of contact in the relationship between the pathogen and the fresh produce is the ability of the pathogen to attach to the fresh produce. After attachment, *L. monocytogenes* survives and grows and it is able to establish itself by successfully colonising the produce through biofilm formation (Patel & Sharma, 2010). The increase in fresh produce related foodborne outbreaks has given rise to researchers developing novel control strategies such as light emitting diodes (LEDs) (Ghate et al., 2017) and aerated steam (Bezanson et al., 2018) to reduce contamination by foodborne pathogens. Confidence in the availability of novel control methods to control foodborne pathogens might reduce consumers concern with pathogen

contamination of food. Studies have examined the use of different washing systems, irradiation methods and biological control methods to minimise pathogen concentration on fresh produce (Olmez & Temur, 2010; Baert et al., 2009; Srey, Jahid, & Ha, 2013). However, none of these methods has been able to completely successful in eliminating foodborne pathogens on fresh produce (Koseki et al., 2004; Seo & Frank, 1999; Ban & Kang, 2008). For example, the number of *L. monocytogenes* on lettuce leaf reduced by 1.6 log CFU/cm^2 when it was treated with 0.1 kGy X-ray at 22 °C (Mahmoud, 2010a). Similarly, *L. monocytogenes* on lettuce leaf reduced by 1.16 log CFU/g when treated with 254 nm UV radiation (3.40mWcm/2) at 4 ℃ for 1 min (Kim et al., 2013). The number of *L. monocytogenes* on lettuce reduced by 0.61 log CFU/g when they were washed with 200 mg/L sodium hypochlorite (pH 5.90–5.95) for 5 min (Baert et al., 2009).

The inability of different control methods to reduce pathogen growth has brought about investigating methods such as hydroponic cultivation. Control strategies involving the use of hydroponic systems for fresh produce cultivation have been reported as a cleaner alternative in their propagation. Hydroponically grown lettuce were found to have a lower risk of microbial contamination as they are grown in controlled environments (Lopez-Galvaz et al., 2014). The results of the previous study revealed differences in the nutrient composition and surface microbiota between hydroponic and soil grown lettuce. Differences in leaf surface microbiota between the two lettuce types might have the potential of affecting pathogen colonisation of produce.

The main objective of this study was to investigate the attachment, survival and growth of *L. monocytogenes* to hydroponic and soil grown lettuce leaves. Another key objective was to show the minimum exposure time for *L. monocytogenes* attachment to both hydroponic and soil grown lettuce. Different researchers (Ijabadeniyi et al., 2010; Engels et al., 2012; Takeuchi

et al., 2000) have investigated the attachment of *L. monocytogenes* to lettuce and other fresh produce but the minimum exposure time which has been reported so far is 5 min (Ells & Hansen, 2006). In this study, the ability of *L. monocytogenes* to strongly attach to hydroponically grown and soil grown lettuce leaves under very short exposure periods of 1s, 10 s, 30 s, 60 s, 2 and 5 min has been demonstrated. The ability of *L. monocytogenes* to survive on both hydroponic and soil grown lettuce leaves for 10 days at 4 and 10°C was also demonstrated. This is the first report describing the rapid attachment of *L. monocytogenes* to lettuce leaf surfaces with minimal exposure times.

5.2 Materials and methods

5.2.1 Lettuce

Lettuces were grown with the same conditions used in Chapter four. Buttercrunch lettuces (*Lactuca sativa* L. *var. capitata*) obtained from Orderings Garden Centres, New Zealand were originally sown from seed, and then maintained on soil potting mix and in hydroponic solution. The growth conditions are described by Sanguandeekul, 1999. Lettuce were four weeks old at time of experimental use. Growth of both hydroponic and soil potting mix lettuces were conducted in a greenhouse with an average temperature of about 20°C at the Plant Growth Unit (PGU), Massey University. Electrical Conductivity (EC) of the nutrient solutions used for the hydroponic solution was maintained between 1.2 and 1.3 dS/m and the pH was controlled daily to ensure it was maintained at 5.8. For the soil potting mix, lettuce seeds were sown thinly in rows with 35 cm apart to cover a depth of 5mm (Sanguandeekul, 1999).

5.2.2 Bacterial strains and inoculum preparation

To ensure relevant strains were used, three different strains of *L. monocytogenes* that had been isolated from a fresh produce environment were chosen. These strains were obtained from the New Zealand Institute for Plants & Food Research Limited (PFR) culture collection. Details of the cultures are shown in Table 5.1. *L. monocytogenes* PFR O8A06 (coleslaw isolate), *L. monocytogenes* PFR O8A07 and *L. monocytogenes* PFR O8A08 (cabbage isolates) were maintained at −80 °C on Brain Heart Infusion (BHI) Broth (Bacto™ Brain Heart Infusion, Becton, Dickinson & Company, Le Pont de Claix, France) and 20% (v/v) glycerol. The frozen culture was first activated in BHI broth at 30 °C for 12–14 h with agitation at 120 rpm (Gallery Orbitron Shaker, INFORS HT, Germany) and then 1:900 sub-cultured in BHI broth for an additional 12–14 h at 30 °C before use.

After culturing twice in BHI, cultures were centrifuged at 4400 x g for 10 min at room temperature (Eppendorf Centrifuge 5702, Hamburg, Germany). The resultant pellet was washed once with Phosphate Buffered Saline (PBS, Code OPM343, Fort Richard Laboratories, Auckland), then resuspended in 0.1% sterile Buffered Peptone Water (BPW, GranuCult™, Merck KGaA, Billerica MA, USA). The culture was serially diluted in BPW to achieve a final cell number of approximately 10^5 CFU/ml which was used as the initial inoculum. For the survival and growth studies, a 2 L pool of inoculum was prepared from the stock culture to obtain a final bacterial load of 10^7 CFU/ml.

Table 5.1. Details of the New Zealand strains of *Listeria monocytogenes* used in this study

Strain Identity	Source	Region	Isolation Year	Genebank Accession Number[1]	Serotype	Lineage	MLST[2] Clonal Complex	MLST Sequence Type
PFR O8A06	Retail Coleslaw	Auckland	1997	FUII01	1/2a	II	8	120
PFR O8A07	Cabbage	Otago	1999	UZBE01	4b	I	2	2
PFR O8A08	Cabbage	Otago	1999	UZBC01	1/2a	II	8	120

[1]Whole genome sequence analysis. [2]Multi Locus Sequence Typing

5.2.3 Inoculation and enumeration of *L. monocytogenes* attachment on lettuce leaf surfaces

Lettuce leaves were cut into 2 cm by 2 cm squares with a sterile cutter and rinsed in 250 ml sterile distilled water with an approximate flow rate of 0.05 L/s for 5s. They were aseptically placed in sterile petri dishes and left to dry under the laminar flow cabinet for 30 min. To inoculate the surface of the lettuce leaves, 500 µL of the inoculum was gently released onto each square to cover the whole surface. The inoculum was kept on the leaf surface for 1 s, 10 s, 30 s, 60 s, 2 min and 5 min for attachment. The leaves were then gently picked with a pair of sterile forceps and washed under running sterile distilled water for 1 min to remove unattached cells.

Enumeration was done by aseptically placing the 2 cm by 2 cm leaf squares into 9 ml 0.1% sterile peptone water filled with 10 g of glass beads. This was mixed by vortex for 2 min to release bacteria from the leaf surface. Serial 10-fold dilutions were spread onto Palcam agar (Code 1440, Fort Richard Laboratories, Auckland). Plates were then incubated for 48 h at 30°C and typical greenish-black colonies counted to quantify *L. monocytogenes* populations.

5.2.4 Inoculation and enumeration of *L. monocytogenes* survival and growth on lettuce leaf surfaces

Twenty-five g of lettuce leaves were submerged in the 2 L *L. monocytogenes* inoculum for 2 min. Lettuce leaves were air dried under a laminar flow cabinet at room temperature for 2 h. The initial inoculum concentration of *L. monocytogenes* on dried lettuce samples was determined by adding 225 ml of 0.1% peptone water and pummelled using a peristaltic blender, Smasher™ Lab Blender (AES-Chemunix) for 120 s at a speed of 250 rpm for 2 min. Homogenates were serially diluted with 0.1% sterile buffered peptone water and plated on Palcam agar (Code 1440, Fort Richard Laboratories, Auckland). The plates were incubated at

30°C for 48 h and colonies typical of *L. monocytogenes* were counted. A final bacterial load of approximately 5 log CFU/g was found on each leaf and this number was used as the initial inoculum concentration. The survival of *L. monocytogenes* was investigated on both bagged and un-bagged lettuce. For the un-bagged lettuce, only *L. monocytogenes* O8A06 strain was investigated. Three hydroponic and three soil grown lettuce samples (25 g each) were stored on a sterile plastic tray (Tote tray, 390 mm L x 270 mm W x 75 mm H; Stowers, New Zealand) at 4 and 10°C. For the bagged lettuce, three hydroponic and three soil grown lettuce leaves were aseptically stored in separate plastic bags (OfficeMax® resealable bags, 100 x 130 mm; China) at 4 and 10°C for 10 days. Enumeration was carried out at 48 h intervals. For each time point at each temperature, 3 independent replicates were enumerated and one negative control (uninoculated lettuce sample) per temperature was analysed.

5.2.5 Statistical analysis

The numbers of *L. monocytogenes* cells attached to the leaves were converted to log CFU/cm^2 and numbers of *L. monocytogenes* cells recovered from lettuce leaves were converted to log CFU/g for the survival and growth studies. For each data point, the standard deviation (SD) and average value were presented as mean ± SD with SD error bars. The experiments were done with three biological repeats with each composing of three technical repeats. Analysis of Variance (ANOVA) and Tukey's test were performed at a 95% confidence level ($p < 0.05$) using Minitab Statistical Software (Minitab Version 17, State College, Pennsylvania, USA) to determine the significant differences between the number of *L. monocytogenes* cells attached and survived for each strain.

5.3 Results and discussion

5.3.1 Attachment of *L. monocytogenes* to hydroponic and soil grown lettuce leaves

The results for all three strains of *L. monocytogenes* exposed to both hydroponic and soil lettuce leaves for different time periods are shown in Figs. 5.1 and 5.2. There was no significant difference between the attachment to hydroponic or soil grown leaves ($p > 0.05$). Attachment occurred within 1 s exposure for all strains. The number of *L. monocytogenes* O8A08 increased slowly with increasing time of exposure. The highest amount of attachment was recorded for strain 08A08 at 2 and 5 min exposure to lettuce leaves grown hydroponically. This was significantly different from the other two strains tested ($p < 0.05$).

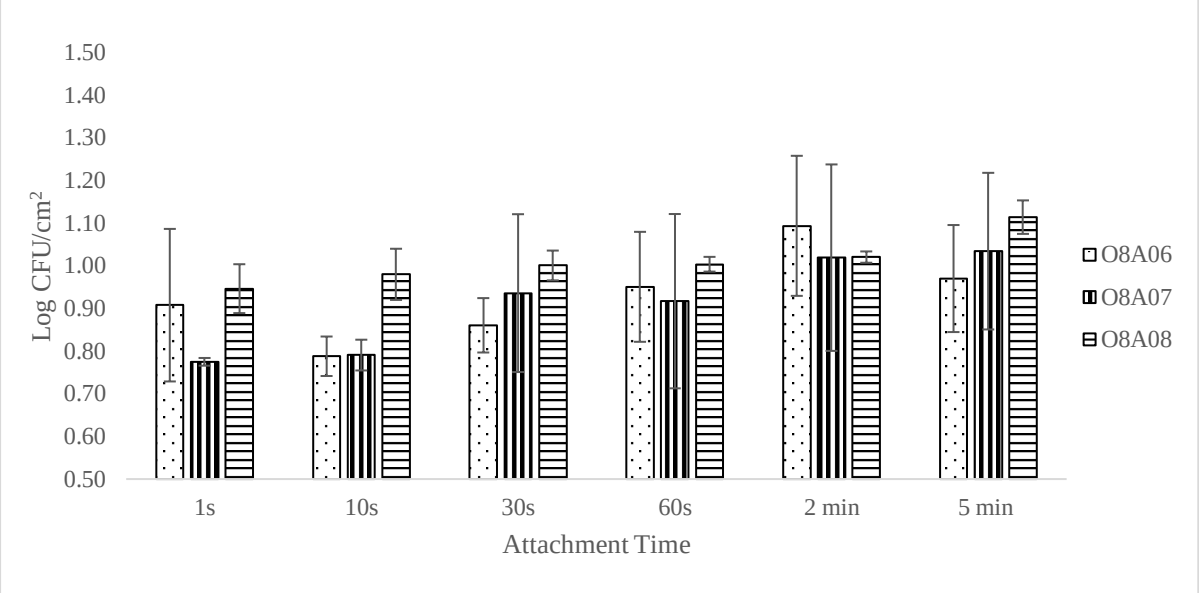


Figure 5.1. Attachment (log CFU/cm^2) of *L. monocytogenes* O8A06, O8A07 & O8A08 on soil grown lettuce leaves at attachment times of 1 s, 10 s, 30 s, 60 s, 2 min and 5 min. Data is represented by the mean of three biological repeats with standard deviation error bars. Statistical differences were performed with ANOVA and Tukey's test at $p < 0.05$.

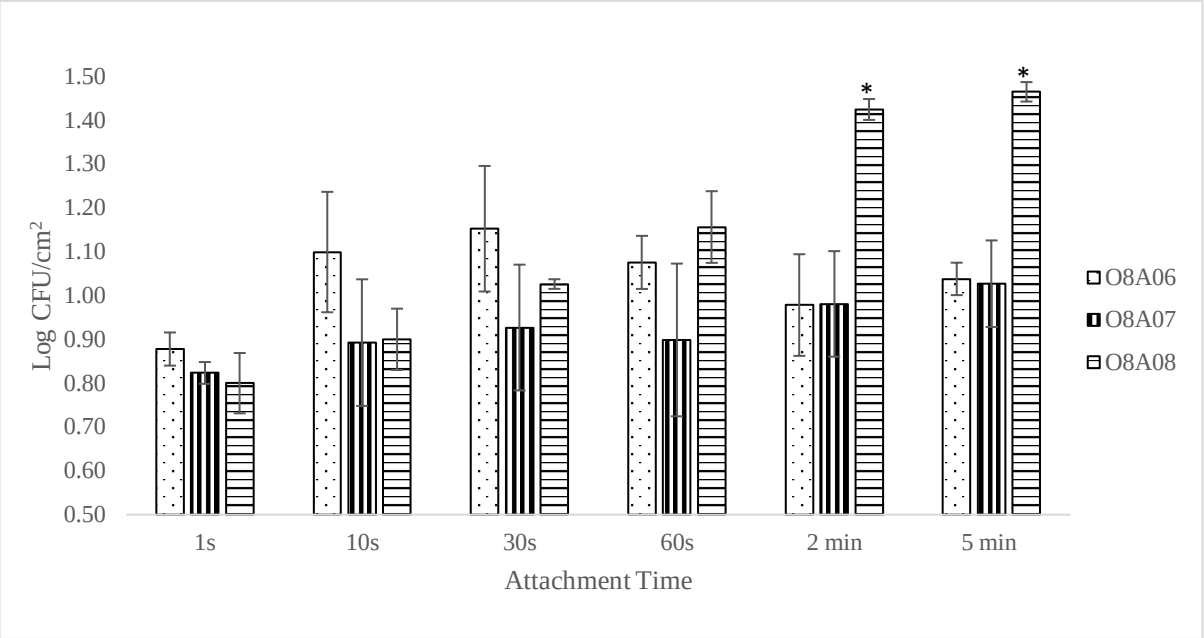


Figure 5.2. Attachment (log CFU/cm^2) of *L. monocytogenes* O8A06, O8A07 & O8A08 on hydroponically grown lettuce leaves at attachment times of 1 s, 10 s, 30 s, 60 s, 2 min and 5 min. Data is represented by the mean of three biological repeats with standard deviation error bars. Statistical differences were performed with ANOVA and Tukey's test at $p<0.05$. Bars with an asterisk (*) denote a significant difference between the strains.

In this study, the attachment of three different fresh produce associated *L. monocytogenes* strains to lettuce leaves was evaluated under minimal exposure periods, shorter than those previously reported (Ijabadeniyi et al., 2010; Engels et al., 2012; Takeuchi et al., 2000). The ability of all the three strains to rapidly attach to surfaces of both hydroponically grown and soil grown lettuce was demonstrated.

Lettuces obtained from different growth systems (hydroponic & soil) were used for this experiment since Selma et al. (2012) has shown that the source (hydroponic and soil) of fresh produce affects the microbiological quality of the produce. They demonstrated this by using three different lettuce types: lollo rosso, red oak and butterhead. They tested for the presence of lactic acid bacteria and total coliforms. They found that hydroponically grown lettuce was 3 log CFU/g and 1.5 log CFU/g lower than soil grown lettuce for lactic acid bacteria and coliforms respectively for all lettuce genotypes tested. They explained that the lack of soil

contaminants might be responsible for the low numbers of coliforms found. High numbers of coliforms correlates to a greater chance of pathogen contamination (USA Environmental Protection Agency, 2006). The results in the previous chapter indicated differences in the microbiomes of hydroponic and soil grown lettuces which suggested that hydroponic grown lettuce may have a higher colonisation resistance to pathogens. However, the results in this chapter were different in that there was no overall significant difference ($p > 0.05$) between the hydroponic and soil grown leaves in terms of the attachment abilities of the *L. monocytogenes* strains that were used. However, strain O8A08 did show significantly ($p < 0.05$) higher attachment to the hydroponically grown leaves than the soil grown ones after the longer exposure periods (2 and 5 min, Figs. 5.1 and 5.2), the opposite of the expected results as well as the finding of Selma et al., (2012). The ability of *L. monocytogenes* to attach to a leaf surface depends on a complex interaction between the bacteria and the leaf and this interaction involves many factors (Kyere et al., 2019). These factors have been found to include both bacterial properties such as cell surface appendages, cell surface charge, outer membrane proteins, extracellular polysaccharides, hydrophobicity and properties associated with the leaf such as leaf age, source of leaf, leaf topography and architecture and leaf surface microflora (Gorski et al., 2009; Brandl & Amundson, 2008; Selma et al., 2012; Kyere et al., 2019). This complexity in bacteria-leaf association may be the reason why my results were different from what other researchers have reported.

Exposure of lettuce to 5 log CFU/ml *L. monocytogenes* for just 1 s resulted in at least 0.77 log CFU/cm^2 attachment while by 2 min this had risen to 1.4 log CFU/cm^2 for strain O8A08. The other two strains only reached 1 log CFU/cm^2. Other researchers have also studied the attachment of *L. monocytogenes* to fresh produce under various exposure periods (Engels et al., 2012; Ijabadeniyi et al., 2010; Takeuchi et al., 2000). Ukuku & Fett, 2002 reported that exposure of cantaloupe to 8 log CFU/ml *L. monocytogenes* H7778 for 10 min resulted in 3.20

log CFU/cm^2 attaching. Note that their inoculum level was approximately 3.0 log CFU/ml greater than mine therefore if the attachment level is proportional to the initial inoculum, the results from the present trial are similar to theirs. In a similar attachment study, 4 log CFU/cm^2 *L. monocytogenes* Scott A attached to cabbage after it was exposed to a concentration of 6 log CFU/ml for 5 min at 22 °C (Ells & Hansen, 2006).

The results indicate that *L. monocytogenes* O8A08 attachment to hydroponically grown lettuce was significantly higher ($p < 0.05$) than O8A06 and O8A07 during 2 and 5 min exposure (Fig. 5.2). The high number observed for O8A08 may be due to the strain used since different strains have different attachment abilities (Ells & Hansen, 2006). Initial attachment of *L. monocytogenes* has been attributed to its movement via the formation of actin-based filaments on the flagella which act as surface adhesins (Takeuchi et al., 2000). This may aid in their rapid attachment to leaf surfaces.

This study reflected a time-independent attachment of *L. monocytogenes* strains O8A06 and O8A07 to lettuce leaves unlike reports from others (Ijabendeniyi et al., 2010; Ells and Hansen, 2006). However, *L. monocytogenes* O8A08 showed a time dependent attachment to both soil and hydroponically grown lettuce leaves. Interestingly, the attachment of *L. monocytogenes* O8A08 to hydroponically grown lettuce leaves during 60 s, 2 min and 5 min exposure times was significantly higher ($p < 0.05$) than soil grown leaves. The results are contradictory to other studies which found that the use of cleaner growing techniques such as hydroponic systems for fresh produce production gave a lower risk of microbial colonisation (Lopez- Galvez et al., 2014). Lima et al. (2003) reported on the effect of leaf type on bacteria colonisation. After 24 h exposure time, they found that *Salmonella* Enteritidis had stronger attachment to leaves from hydroponically grown lettuce than soil grown lettuce due to the differences in leaf surface

hydrophobicity. There have been very few studies about the effect of leaves produced by different growth systems on pathogen colonisation.

To the best of our knowledge, there have been no studies on the relationship between the source of fresh produce and *Listeria* colonisation and therefore, investigations will be necessary in our quest to understand fresh produce pathogen interaction and control.

5.3.2 Survival of *L. monocytogenes* to hydroponic and soil grown lettuce leaves

L. monocytogenes population decreased by 3.5 log CFU/g from the starting inoculum concentration in both un-bagged hydroponic and soil grown lettuces at 10°C after 240 h (Fig 5.3). The *L. monocytogenes* population decreased below the detection limit in both un-bagged hydroponic and soil grown lettuces at 4°C after 240 h (Fig 5.4). The results agree with the previous results about the prevalence of *L. monocytogenes* in bagged and un-bagged lettuces sold in supermarkets. Exposing fresh produce to air has been reported to reduce the *L. monocytogenes* population (Bardsley et al., 2019). Bardsley et al. (2019) reported a 2.4 log decrease in *L. monocytogenes* on field grown basil within 5 h. There was no significant difference ($p > 0.05$) in *L. monocytogenes* O8A06 survival in un-bagged hydroponic and soil grown lettuce. Since there were no significant growth of *L. monocytogenes* O8A06 on un-bagged lettuce, all further experiments were performed with bagged lettuces.

The survival of all the *L. monocytogenes* strains (O8A06, O8A07 and O8A08) in bagged hydroponic and soil grown lettuce was similar at each temperature. *L. monocytogenes* generally decreased from the t=0 to t=144 h and start to increase from t=144 h at 10°C (Fig 5.3). *L. monocytogenes* at 4°C decreased to t=192 h and then start to grow. *L. monocytogenes* growth for all three strains at 10°C from t=144 to t=240 was between 0.3 to 0.9 log CFU/g however growth from t=192 h to t=240 h was between 0.1 to 0.67 log CFU/g at 4°C (Fig 5.5).

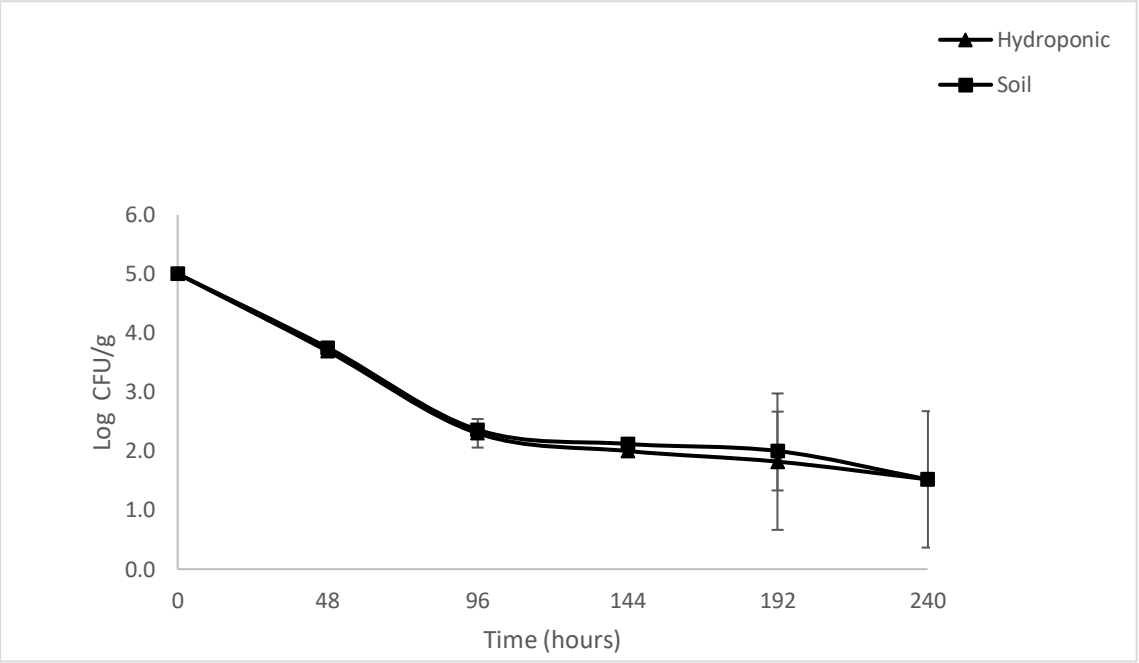


Figure 5.3 Survival of *L. monocytogenes* O8A06 on hydroponic and soil grown un-bagged lettuce leaves at 10°C for t=0 to =240 h. Triangular data points represent hydroponic grown lettuce leaves and rectangular data points represent soil grown lettuce. Data is represented by the mean of three biological repeats with standard deviation error bars. Statistical differences were performed with ANOVA and Tukey's test at $p<0.05$.

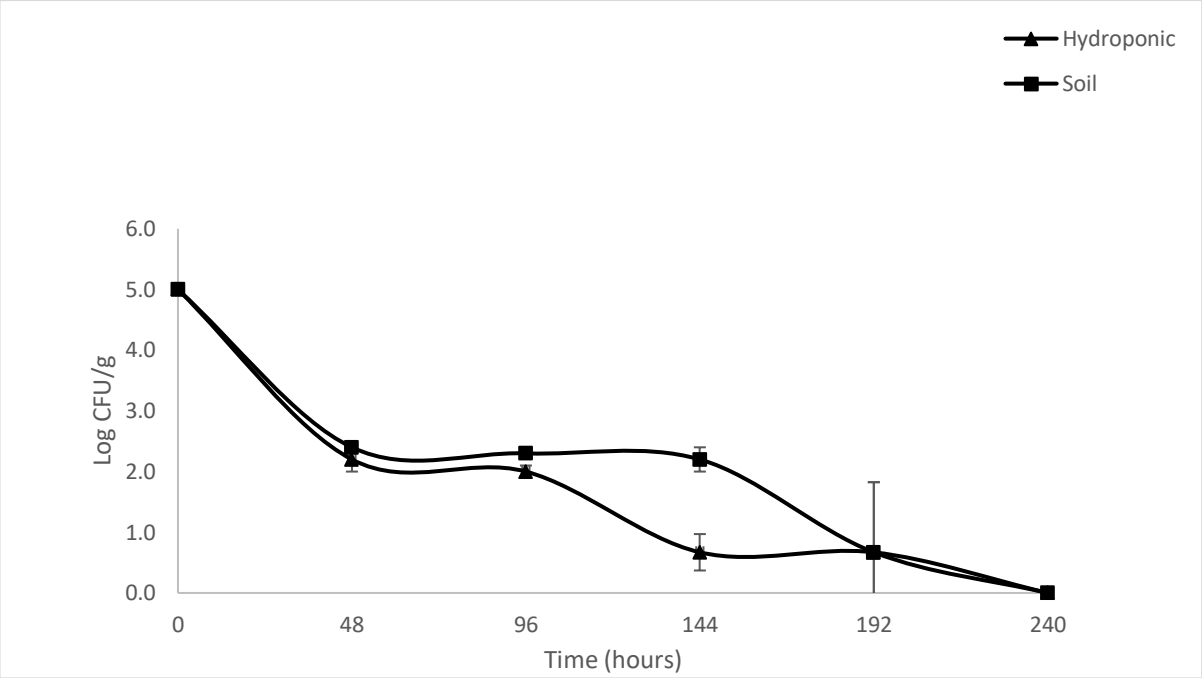


Figure 5.4 Survival of *L. monocytogenes* O8A06 on hydroponic and soil grown un-bagged lettuce leaves at 4°C for t=0 to =240 h. Triangular data points represent hydroponic grown lettuce leaves and rectangular data points represent soil grown lettuce. Data is represented by the mean of three biological repeats with standard deviation error bars. Statistical differences were performed with ANOVA and Tukey's test at $p<0.05$.

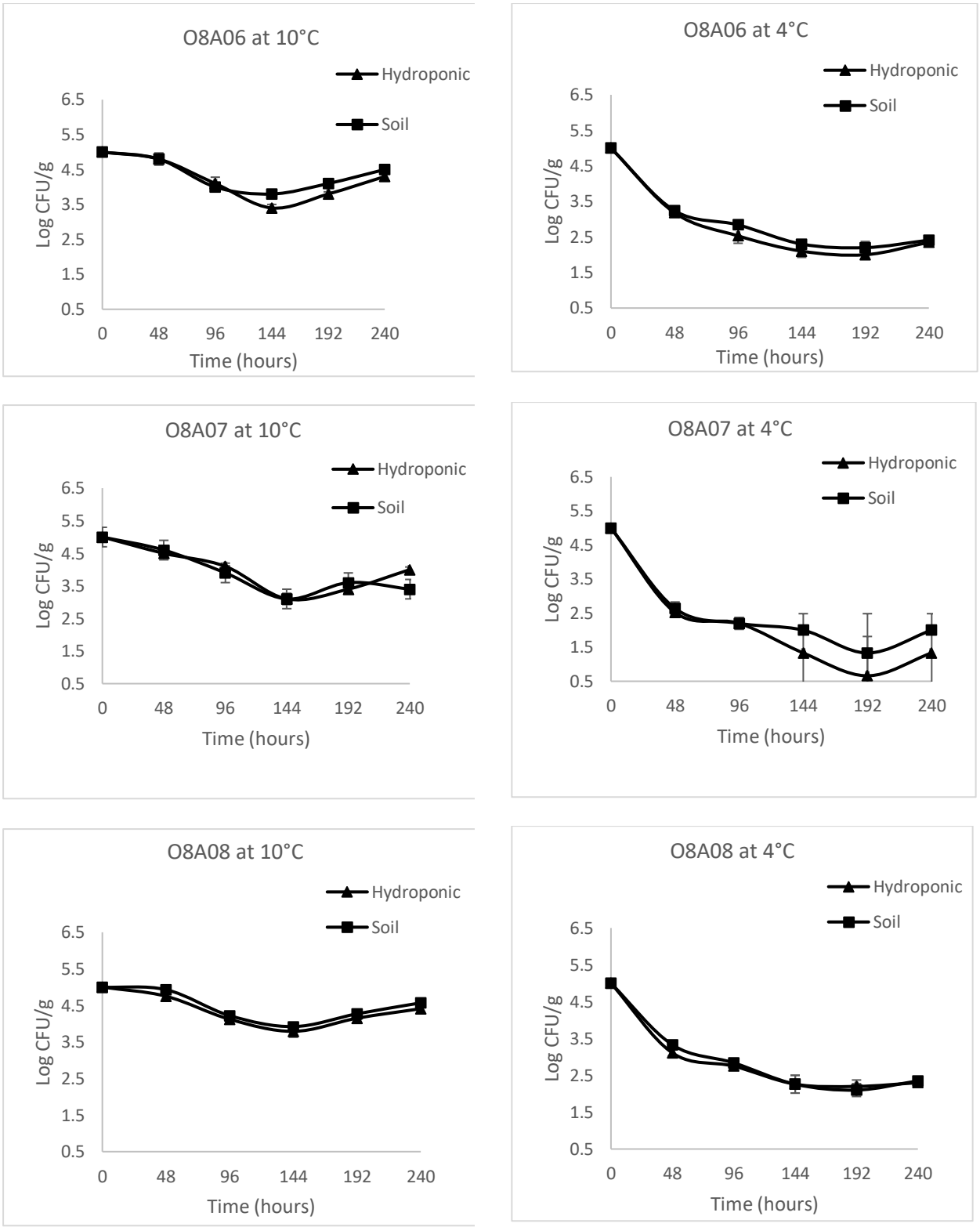


Figure 5.5. Survival and growth of *L. monocytogenes* on hydroponic and soil grown bagged lettuce leaves at 4 and 10°C for t=0 to t=240 hours. Triangular data points represent hydroponic grown lettuce leaves and rectangular data points represent soil grown lettuce. Data is represented by the mean of three biological repeats with standard deviation error bars. Statistical differences were performed with ANOVA and Tukey's test at $p < 0.05$.

The survival of *L. monocytogenes* on fresh produce surfaces has been reported (Likotrafiti et al., 2013). The population of *L. monocytogenes* did not grow on lettuce leaves (stored at 90% relative humidity-RH) at 10°C for seven days. The *L. monocytogenes* population remained unchanged until day four (96 h) and decreased more than 0.5 log before growing from day five (120 h) to the seventh day (168 h) (Likotrafiti et al., 2013). This is similar to the current study where *L. monocytogenes* reduced from t=0 until day six (144 h) and started to grow after day six (144 h) through day 10 (240 h). The *L. monocytogenes* population reduced by 0.43-1.6 log CFU/g after 10 days. The 90 % RH in the Likotrafiti et al. (2013) study might be the reason for the differences in *L. monocytogenes* population between their study and this study. This can further be explained in their second study where they investigated the growth of *L. monocytogenes* on lettuce under the same conditions but with 53% RH instead of 90% RH. They observed a steady decrease in *L. monocytogenes* population until the seventh day with 1 log difference from the initial inoculum concentration. The actual relative humidity in the bagged lettuce samples was not determined. The results of this study suggest the importance of relative humidity in *L. monocytogenes* growth on fresh produce as suggested by other authors (Zoz et al., 2016; Park & Kang, 2015).

The growth of *L. monocytogenes* on iceberg lettuce packaged by MAP (1-7% O_2 and 9-15% CO_2) for 8 days at 5 and 8°C was investigated by Ziegler et al. (2019). *L. monocytogenes* increased by 0.8 log CFU/g at 5°C while there was 1.4 log CFU/g increment after 8 days at 8°C. In the present study, growth of *L. monocytogenes* at 10°C was significantly higher ($p < 0.05$) than growth at 4°C. One of the main factors which influence *L. monocytogenes* growth on fresh produce is temperature and high temperatures have been correlated with higher growth in *L. monocytogenes* (Carrasco et al., 2008; Ding et al., 2010).

5.4 Conclusion

The attachment of *L. monocytogenes* is the first essential step for their survival and growth on the surfaces of lettuce leaves (Shenoy et al, 2017). These results show that any exposure of lettuce leaves to *L. monocytogenes* for even a very short time can result in attachment irrespective of the growth system used. Once exposed for even such a short time, attached bacteria are difficult to remove. After attachment, *L. monocytogenes* can survive and grow on both hydroponic and soil grown lettuce leaf surfaces which may subsequently lead to biofilm formation (Patel & Sharma, 2010). Growth of *L. monocytogenes* is exacerbated in bagged lettuces which might be due to the relative humidity. The results indicate that it is more important to prevent any form of pathogen contamination in fresh produce (Kyere et al., 2019) rather than relying on postharvest food safety control measures to remove them. Any slight exposure of lettuce to *L. monocytogenes* can be a hazard to food safety.

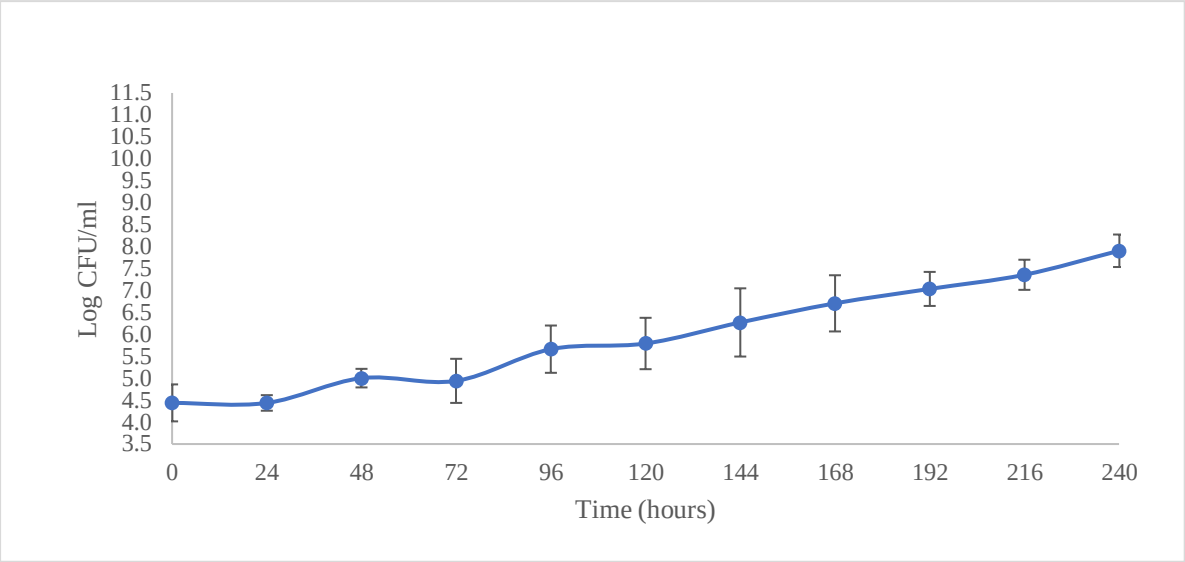
5.5 Summary of chapter and the link to the next chapter

The results of this chapter for the first time have revealed the ability of *L. monocytogenes* to rapidly attach to both hydroponically grown and soil grown lettuce leaves under the most minimum exposure time. Irrespective of the differences (in nutrients content and leaf microbiome) between hydroponic grown and soil grown lettuce leaves which was revealed in the previous chapter, *L. monocytogenes* was able to attach, survive and grow on both lettuce types. Growth of *L. monocytogenes* was better in the bagged environment than in un-bagged environment. In the bagged environment, the growth of *L. monocytogenes* was better at 10°C than at 4°C. At both temperatures, *L. monocytogenes* numbers generally decline from t=0 and start to increase after 6 or 8 days. *L. monocytogenes* survived on both lettuce types at 4 and

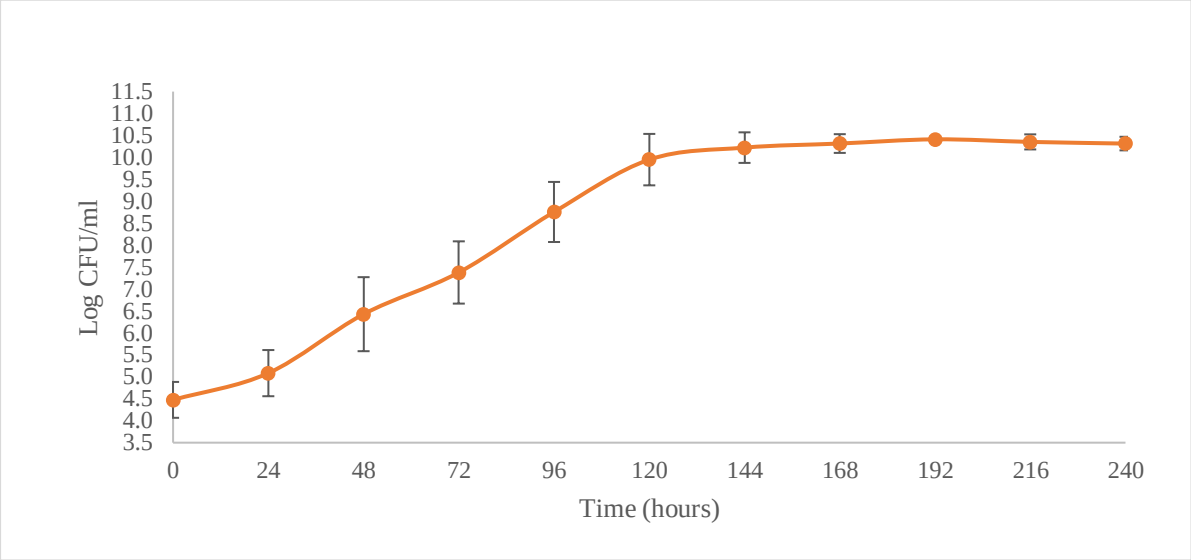
10°C. This indicates that the differences in leaf surface microbiome and nutrient content might not have a direct effect on *L. monocytogenes* attachment, survival and growth.

Another important part of *L. monocytogenes* colonisation is biofilm formation. The mechanisms for bacterial attachment have been found to be different from the mechanisms of biofilm formation (Garrett et al., 2008). Therefore, in the next chapter, the biofilm formation of *L. monocytogenes* in juices from both lettuce types on stainless steel coupons will be investigated. The reason for investigating the biofilm formation of *L. monocytogenes* in lettuce juices is due to its direct application in the fresh produce industry. Information from the literature suggests that many outbreaks have implicated the fresh produce environment as the source of *L. monocytogenes* contamination (McCollum et al., 2013). During a visit to a local fresh produce processing industry, it was revealed that washing, cutting and shredding released juices from lettuce on surfaces during processing. Lastly, internalization of pathogens in fresh produce has been reported (Shenoy et al., 2017). In the next chapter (chapter 5), the ability of *L. monocytogenes* to form biofilms in hydroponic and soil grown lettuce juices (grown under the same conditions in chapter 5) will be investigated with the same *L. monocytogenes* strains used previously. The conditions used in the study were designed to simulate the fresh produce processing environment which is usually operated in cold temperatures. The results will enable us to know whether lettuce leaf extracts can support *L. monocytogenes* biofilm growth. Also, the differences between hydroponic and soil grown lettuce juices in supporting *L. monocytogenes* biofilm growth will be revealed.

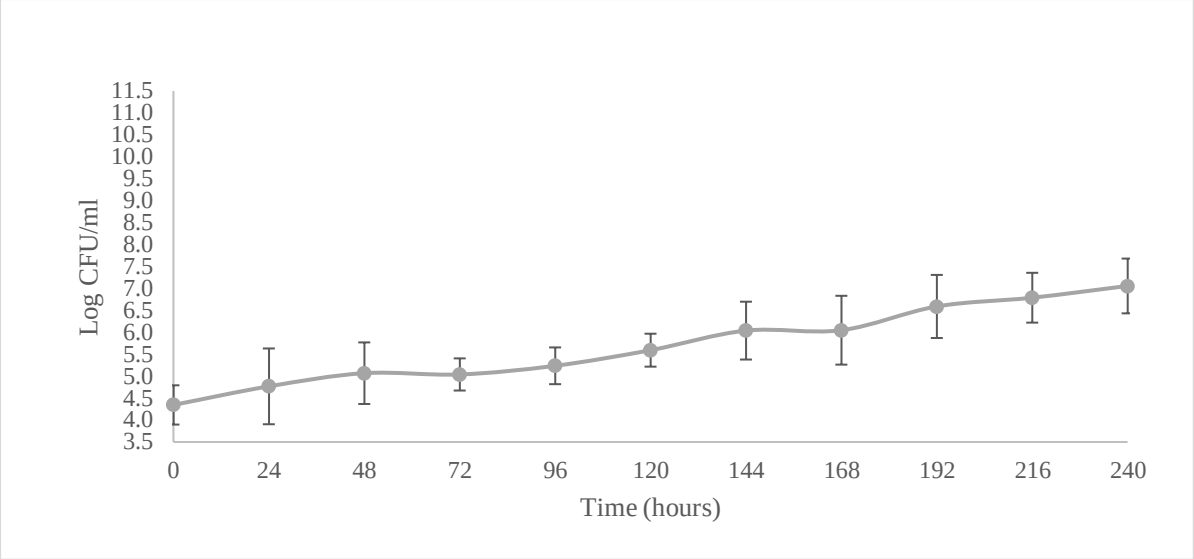
5.6 Supplementary information



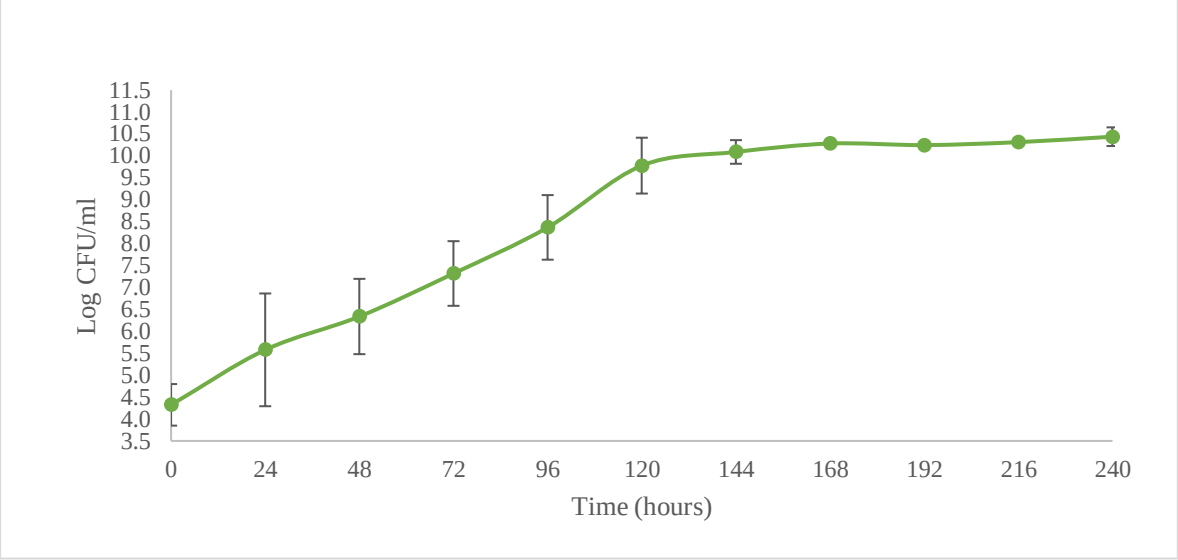
Supplementary Information S5.1. Growth curve of *L. monocytogenes* O8A06 (log CFU/ml) at 4°C in BHI broth for t = 0 to t = 240 h.



Supplementary Information S5.2. Growth Curve of *L. monocytogenes* O8A06 (log CFU/ml) at 10°C in BHI media for t = 0 to t = 240 h.



Supplementary Information S5.3. Growth Curve of *L. monocytogenes* O8A07 (log CFU/ml) at 4 °C in BHI media for t = 0 to t = 240 h.



Supplementary Information S5.4. Growth Curve of *L. monocytogenes* O8A07 (log CFU/ml) at 10 °C in BHI media for t = 0 to t = 240 h.

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CHAPTER 6 Biofilm formation of *Listeria monocytogenes* in hydroponic and soil grown lettuce leaf extracts on stainless steel coupons

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Abstract

Foodborne outbreaks associated with fresh produce due to human pathogenic bacteria have become a global concern. In this study, the ability of three *L. monocytogenes* strains: PFR O8A06 (coleslaw isolate), PFR O8A07 and O8A08 (cabbage isolates) originating from fresh produce, to survive and form biofilms on stainless steel coupons at 4°C and 10°C in extracts of lettuce leaves obtained from soil and hydroponic systems was investigated. There was no significant difference ($p > 0.05$) between the hydroponic and soil grown leaf extracts in terms of *L. monocytogenes* survival, growth and biofilm formation. The biofilm formation of *L. monocytogenes* on stainless steel for both soil and hydroponic leaf extracts at 10°C increased from 3 log to 6.4-7.2 log CFU/cm^2. At 4 °C, the biofilm formation of all three strains for both soil and hydroponic leaf extracts increased to 4.3-4.8 log CFU/cm^2. These findings suggest that lettuce leaf extracts support the survival, growth and biofilm formation of *L. monocytogenes* on stainless steel irrespective of the growth system used and this might be a potential cause for recurring contamination in the processing environment.

6.1 Introduction

There are a number of companies who grow, harvest, process and package lettuce for sale. In the fresh produce processing environment, harvested lettuces are cut, washed and packaged on large scale (FAO, 2010; Holvoet, 2014).

L. monocytogenes has caused many food poisoning outbreaks related to the contamination of fresh produce (Kocot & Olszewska, 2017; Gaul et al, 2010; Shrivastava, 2011). *L. monocytogenes* continues to be involved in many recent fresh produce-related outbreaks (NZFS, 2017; NSW Food Authority, 2018; EFSA, 2018). Some foodborne outbreaks associated with fresh produce due to *L. monocytogenes* have implicated the processing environment as the source of contamination (McCollum et al, 2013). For example, the recent listeriosis outbreak in 2018 involving Australian cantaloupes, which caused five deaths and many illnesses, was associated with *L. monocytogenes* found on the packing floor of the factory (NSW Food Authority, 2018). Investigations to track the source of the European multi-country (Austria, Denmark, Finland, Sweden and UK) *L. monocytogenes* outbreak, which was associated with frozen corn and other vegetables, was traced to a freezing plant in Hungary and the strain was thought to be persisting in the processing environment (EFSA, 2018).

Several studies have described leafy greens as unfavourable media for the survival and growth of *L. monocytogenes* (Jacxsens et al, 1999; Farber et al, 1998) while others have reported the ability of leafy greens to support *L. monocytogenes* growth (Koseki & Isobe, 2005; Omac et al., 2018). A study conducted by Manois et al. (2013) described the growth of *L. monocytogenes* in lettuce extract (obtained by blending with a Waring blender) and sterilized to 80°C. However, it is highly unlikely to find such lettuce extract in this state in a produce processing environment. One aspect that has not been considered is the biofilm growth of *L.*

monocytogenes on the stainless-steel surfaces of lettuce processing plants and the role of lettuce extracts in supporting biofilm formation on stainless steel.

Several researchers have reported the use of cultivation systems, such as hydroponic and aquaponic systems, as important control strategies to produce cleaner and safer fresh produce (Lopez-Galvez et al., 2014; Orozco et al., 2008). A safer fresh produce source is essential to prevent both pre-harvest and post-harvest contamination (Neto et al., 2012). Other researchers have also reported the risk associated with soil grown fresh produce suggesting the diversity of microorganisms including opportunistic pathogens which may be found in the soil as a source (Alegbeleye et al., 2018; James, 2016). Studies conducted by Franz et al. (2007) showed that the number of *E. coli* in lettuce grown in soil was significantly higher ($p < 0.05$) than lettuce grown in hydroponic solution. The differences between soil and hydroponically grown produce in terms of their nutrient content have been investigated. For example, Treftz et al. (2015) evaluated the differences between the nutrient content (ascorbic acid, α-tocopherol and total phenolics) of hydroponic and soil grown strawberries and concluded that the hydroponic grown strawberries were richer in the selected nutrients than soil grown strawberries. In another study, Selma et al. (2012) reported that hydroponically grown fresh-cut lettuce had higher vitamin C and phenolic compounds than soil grown lettuce. Also, the microbiological quality of the hydroponically grown lettuce was higher than soil grown lettuce since lactic acid bacteria and coliform counts for the hydroponic grown lettuce were 3 log and 1.5 log CFU/g lower respectively than for soil grown lettuce. This shows the possibility of differences in nutrients in produce to affect bacterial growth. Their study did not establish any relationship between nutrient content and bacteria count.

In chapter four, the concentrations of sodium, zinc and boron were found to be significantly higher ($p<0.05$) in soil grown lettuce leaves than hydroponic grown lettuce leaves. However,

there were no significant differences ($p > 0.05$) in other nutrients (iron, manganese, copper, nitrogen, phosphorus, potassium, sulphur, calcium and magnesium) between hydroponic grown and soil grown lettuce leaves. Also, there was no significant differences ($p > 0.05$) between *L. monocytogenes* attachment to hydroponic and soil grown lettuce under minimal exposure times (Kyere et al 2019). However, since the mechanisms of bacterial attachment have been found to be different from growth and biofilm formation (Garrett et al., 2008), it was imperative to investigate the differences between soil and hydroponically grown lettuce extracts with respect to *L. monocytogenes* survival, growth and biofilm formation.

The conditions used in this study were designed to simulate the fresh produce processing environment which is usually operated in cold temperatures. The most ideal temperature required for the fresh produce would be 4°C or below but in reality, temperatures of up to about 10°C are common in processing facilities (Mercier et al., 2017), therefore, I used 4°C and 10°C in my experiments. In the fresh produce environment, continuous washing, cutting and trimming results in the release of leafy green extracts on surfaces (FAO, 2010). Stainless steel is widely used for the equipment used in the fresh produce industry, so when washing and cutting is done there is a continuous contact of fresh produce with stainless steel surfaces. In some processing lines, the rinse water is recirculated and reused for washing or for other operations (FAO, 2010).

The ability of *L. monocytogenes* to attach and form biofilms on stainless steel is a concern in terms of food safety (Kocot et al., 2017), however no studies have been done to investigate the potential of hydroponic and soil grown lettuces extracts to support *L. monocytogenes* biofilm formation on stainless steel. The purpose of this study was therefore to evaluate and compare the biofilm formation of *L. monocytogenes* on stainless steel coupons with lettuce extract

obtained from both soil and hydroponic growth systems simulating conditions prevalent in fresh produce processing facilities.

6.2 Materials and Methods:

6.2.1 Lettuce

Buttercrunch lettuce seeds (*Lactuca sativa* L. *var. capitata*) were grown under the same conditions as described by Kyere et al. (2019). Lettuce plants were four weeks old at time of the experiments. An average temperature of 20 °C was maintained in the greenhouse in the Plant Growth Unit of Massey University, Palmerston North. The hydroponic solution was maintained at Electrical Conductivity (EC) values between 1.2 to 1.3 dS/m and a pH of 5.8. Lettuce seeds were sown thinly in rows which were 35 cm apart at a depth of 5 mm for the soil potting mix (Sanguandeekul, 1999).

6.2.2 Bacterial Strains and Inoculum Preparation

The inoculum preparation used by Kyere et al. (2019) was followed. Three different isolates of *L. monocytogenes* were obtained from the New Zealand Institute for Plant & Food Research Limited (PFR) culture collection. *L. monocytogenes* O8A06 (coleslaw isolate), *L. monocytogenes* O8A07 and *L. monocytogenes* O8A08 (cabbage isolates). *L. monocytogenes* O8A06 and O8A08 had similar MLST sequence types and belong to the same serotype but were isolated two years apart from different products in different geographic locations (Nowak et al., 2017). Stock cultures were maintained at -80 °C in the form of protective beads on Brain Heart Infusion (BHI) Broth (Bacto™ Brain Heart Infusion, Becton, Dickinson & Company, Le Pont de Claix, France) and 20 % (v/v) glycerol. Frozen culture was first activated in BHI broth at 30°C for 12 – 14 h with agitation at 120 rpm (Gallery Orbitron Shaker, INFORS HT,

Germany) and then 1:900 sub-cultured in BHI broth for an additional 12 – 14 h at 30°C before use.

Isolates were cultured twice in BHI and cells were harvested by centrifugation at 4400 g for 10 min at room temperature (Eppendorf Centrifuge 5702, Hamburg, Germany). The resultant pellet was washed once with Phosphate Buffered Saline (PBS, Code OPM343, Fort Richard Laboratories, Auckland) and then dissolved in 0.1 % sterile Buffered Peptone Water (BPW, GranuCult™, Merck KGaA, Billerica MA, USA). Serial dilutions were done in BPW to obtain a final cell count of approximately 10^5 CFU/ml which was used as the initial inoculum.

6.2.3 Preparation of lettuce extract for *Listeria* inoculation and survival studies

Lettuce leaves were aseptically harvested with sterile scissors and aseptically transported in 4°C cold storage boxes to the Massey University food microbiology laboratory, Palmerston North. Leaves were washed with sterile distilled water to remove contaminants such as soil and insects. They were then left to dry in a laminar flow cabinet for 30 min. 10 g of soil or hydroponically grown lettuce leaves were diluted with 9x sterile distilled water in a sterile stomacher bag. This was put in a peristaltic blender (Blender SMASHER®, Biomerieux, Marcy-l'Étoile, France) for 60 s on slow mode. This setting gave the closest representation of lettuce juice been released during washing on a processing line. The resultant mixture was named as leaf extract. Lettuce extracts were serially diluted and plated on Palcam agar (Code 1440, Fort Richard Laboratories, Auckland) to ensure there was no contamination with *L. monocytogenes*. The extract was transferred into 250 ml sterile conical flasks, inoculated with 5 $\log_{10}$ of the *L. monocytogenes* cocktail and swirled with agitation at 80 rpm (Gallery Orbitron Shaker, INFORS HT, Germany) for 2 min to ensure a uniform mix. Since I was interested in *L.* monocytogenes ability to form biofilms in the presence of any other potential microorganisms that could be present, I did not sterilise our extract. Sterile distilled water

inoculated with *L. monocytogenes* was used as control. The flasks were stored at 4°C and 10°C for 240 h. Plating on Palcam agar (Code 1440, Fort Richard Laboratories, Auckland) was done every 48 h from t= 0 h to t= 240 h. pH values for all leaf extracts were recorded with a Mettler Toledo S220 SevenCompact pH/Ion benchtop meter, (Fisher Scientific, New Zealand) before they were enumerated for *L. monocytogenes* growth. Three biological repeats with three technical repeats each were carried out.

6.2.4 Biofilm formation

Biofilm formation was done according to the method described by Zain et al. (2017). Five ml of the leaf extract was transferred into the wells of a 6-well tissue culture plate (Polystyrene Microplates™, Falcon®, Durham, USA). The plates were then inoculated with 5 log *L. monocytogenes* into each well. 5 ml of sterile distilled water was used as the control. The 6-well tissue culture plates were shaken for 2 min at 80 rpm to ensure a uniform mix. A 1cm by 1 cm sterile stainless steel (304 stainless steel with a 2B surface finish) coupon was then placed into each well using a pair of sterile forceps. The microtiter plate was stored at 4°C and 10°C. At 48 h time intervals, 3 metallic coupons for each sample were picked with a pair of sterile forceps and rinsed with 1 ml of sterile distilled water three times on each side of the coupon to remove unattached cells. Each stainless steel coupon was then transferred into 25 ml glass bottles containing 9 ml 0.1% BPW and 10 g of sterile glass beads. The bottles were mixed by vortex for 2 min to release attached *L. monocytogenes* biofilm cells. Appropriate serial dilutions were done, plated on Palcam agar and incubated at 30 °C for 48 h. Three biological repeats with three technical repeats were carried out. To confirm the biofilms formed, scanning electron microscope (SEM) images of *L. monocytogenes* biofilms formed on stainless steel coupons were also taken.

6.2.5 Scanning Electron Microscopy

The biofilm formation of *L. monocytogenes* on stainless steel coupons with lettuce extract as medium was confirmed by SEM. The coupons were sent to the Manawatu Microscopy and Imaging Center, Massey University, Palmerston North for imaging after each sampling time. The coupons were prepared for scanning electron microscopy (SEM) as described by Lindsay & von Holy (1999). Coupons were put in a fixative prepared with glutaraldehyde (3 %) and formaldehyde (2%) in a 0.1 M phosphate buffer immediately on receipt. They were left in the fixative for at least 12 h before proceeding with processing. Samples were then washed with 0.1 M phosphate buffer three times with 15 min incubation at each step. The phosphate buffer (pH 7.2) was replaced with 25 % ethanol and incubated for 15 min. After that, the 25 % ethanol was discarded and samples were sequentially incubated with 50 %, 75 %, 95 % and 100 % ethanol for 15 min at each step. Samples were then dried with liquid CO_2 and placed on aluminium stubs. Finally, they were coated with gold for 200 s and viewed under the FEI Quanta 200 Environmental scanning electron microscope (FEI Company, Hillsboro, OR) at 20 kV.

6.2.6 Statistical Analysis:

The numbers of *L. monocytogenes* cells recovered from lettuce leaves were converted to $\log_{10}$ CFU/g for the survival and growth studies and $\log_{10}$ CFU/cm^2 for biofilm formation. The experiments were done with three biological repeats with each comprising three technical repeats. All mean values were calculated from the results of the three biological repeats with triplicate samples. Minitab Statistical Software (Minitab Version 17, State College, Pennsylvania, USA) was used for analysis of variance (ANOVA) using Tukey's test at a 95% confidence ($p < 0.05$) to determine the significant differences between the number of *L. monocytogenes* recovered from lettuce extracts or stainless steel coupons.

6.3 Results & Discussion:

6.3.1 *L. monocytogenes* survival and growth in lettuce leaf extracts

The growth of all the *L. monocytogenes* strains (O8A06, O8A07 and O8A08) in both soil and hydroponic lettuce extract was similar at each temperature (Fig. 6.1). *L. monocytogenes* numbers grew to about 10 $\log_{10}$ CFU/g for both soil and hydroponic grown lettuce at 10°C. Similarly, *L. monocytogenes* numbers grew to about 7-8 log for both systems at 4°C after 240 h. When they differed, the number of *L. monocytogenes* growth in soil leaf extract was higher than hydroponic leaf extract even though the differences were not statistically significant ($p > 0.05$). All *L. monocytogenes* strains at 10°C had grown to stationary phase after 144 h whereas at 4°C they had not reached stationary phase after 240 h. The number of *L. monocytogenes* in sterile distilled water (used as a control) did not increase but decreased with time dropping below the detection limit by 192 h, often earlier. This indicates that both soil and hydroponically grown lettuce leaf extracts supported the growth of *L. monocytogenes* at 10°C. The number observed in the soil leaf extracts was always higher than hydroponic leaf extracts even though the difference was not statistically significant. There was no significant difference ($p > 0.05$) between the three strains in terms of their growth at 4°C (Fig. 6.1). The pH of both soil and hydroponic leaf extracts decreased gradually with time, dropping from pH 6.5 to 4.5 which may be due to the increase in the number of *L. monocytogenes* in the lettuce extract. A higher number of bacteria results in greater consumption of nutrients by the bacteria, creating an acidic environment with a decrease in pH (Muller, 2010).

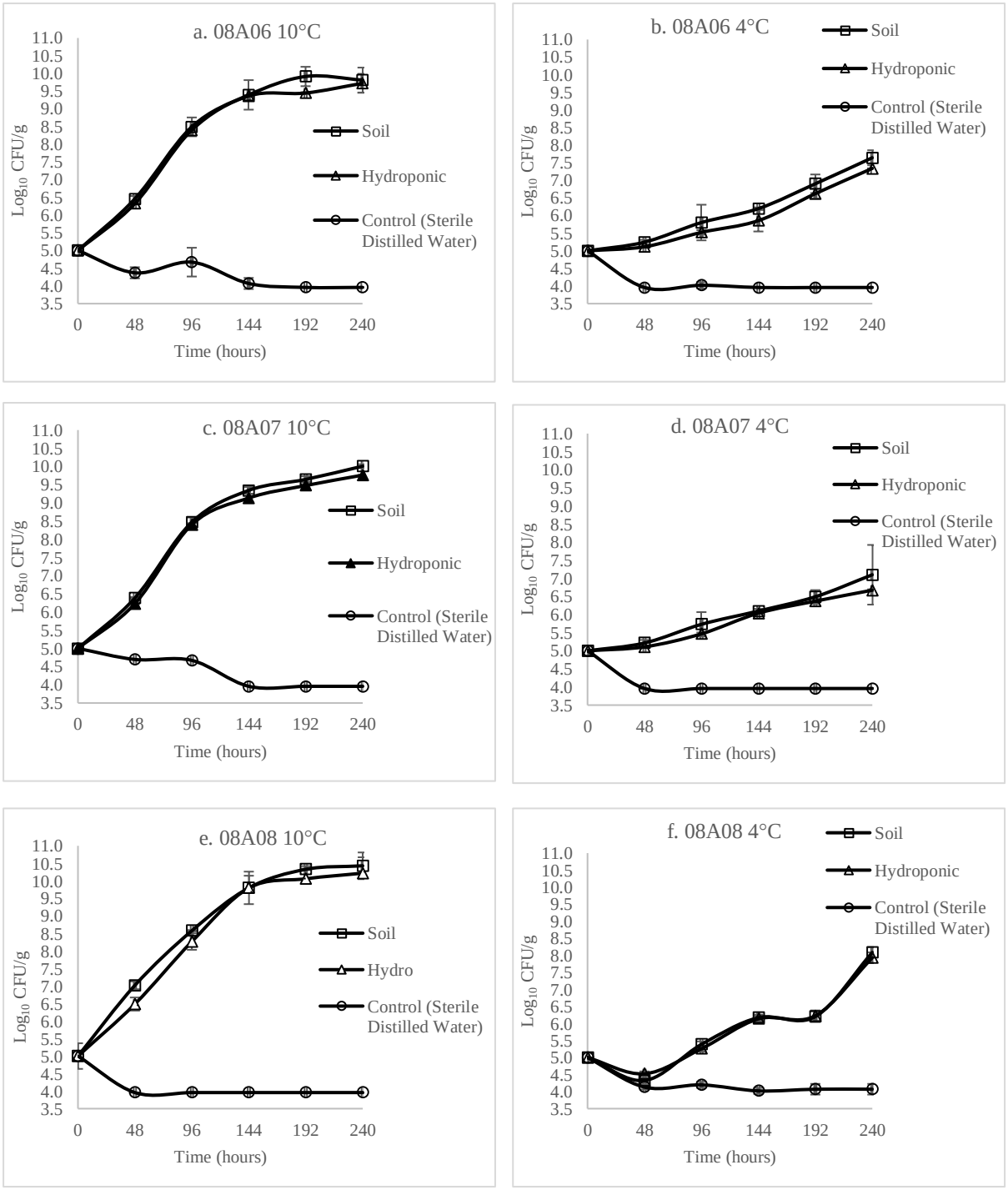


Figure 6.1. Survival and growth of *L. monocytogenes* O8A06, O8A07 & O8A08 (log CFU/g) in control (sterile distilled water), soil and hydroponically grown lettuce leaves at 4°C & 10°C for t = 0 to t = 240 represented by the mean of three biological repeats with standard deviation error bars. Statistical differences were performed with ANOVA and Tukey's test at $p < 0.05$.

Several studies have observed the growth of *L. monocytogenes* and other human pathogenic bacteria on fresh produce (Omac et al, 2018; Koseki & Isobe, 2005; Farber et al., 1998). A study conducted by Koseki & Isobe (2005), to determine the growth of *L. monocytogenes* on iceberg lettuce leaf surface at 5 and 10°C revealed that *L. monocytogenes* population increased by 1.95 log CFU/g at 5°C and 2.08 log CFU/g at 10°C after 92 h and 44 h respectively. My results from homogenised lettuce leaves showed that *L. monocytogenes* growth after 240 h at 4°C was 2 log more than the starting inoculum (Fig. 6.1) while the population at 10°C was 4 log more than the initial inoculum. This was more than that found by Koseki & Isobe (2005) as they only stored their leaves for 20 h and my process of obtaining the lettuce extract resulted in more disruption than their lettuce surfaces that may have resulted in greater availability of nutrients for microbial growth. Ziegler et al. (2019) observed 2.23 log CFU growth after 8 days at 8°C when *L. monocytogenes* was inoculated on iceberg lettuce stored under a modified atmosphere. They concluded that one of the main factors for *L. monocytogenes* growth in fresh produce might be the availability of plant juice which depends on how the vegetable was cut. On the other hand, Delaquis et al. (2006), reported the inability of aqueous extracts prepared from shredded iceberg lettuce to support *L. monocytogenes* growth at 15°C. They attributed this reduction to a potential antilisterial inhibitor which may be present in shredded lettuce. The present study compared if hydroponic and soil grown lettuce extract could support *L. monocytogenes* growth. From the results, it is evident that, both lettuce extracts are able to support *L. monocytogenes* growth contrary to what Delaquis et al. (2006) found and even better than growth on lettuce surfaces found previously and has also been reported by others (Ziegler et al., 2019). Moreover, internalization of pathogenic bacteria in fresh produce, reported by Shenoy et al. (2017) is an indication that producers should not only consider *L. monocytogenes* growth on fresh produce surfaces but also look to processing control measures in order to reduce contamination with vegetables juices obtained during processing.

6.3.2 Biofilm formation of *L. monocytogenes* on stainless steel coupons using lettuce leaf extracts as media

From the results, both soil and hydroponic leaf extracts supported the biofilm formation of all the strains (Fig. 6.2) on stainless steel. There was no significant difference ($p > 0.05$) in *L. monocytogenes* biofilm formation between the soil and hydroponic grown lettuce extracts. The biofilm formation of *L. monocytogenes* after 240 h at 10°C was about 3 log higher than biofilms formed at 4°C. Many researchers (Reis-Teixeira et al., 2017; Oliveira et al., 2010; Takahashi et al., 2009) have reported the biofilm formation of *L. monocytogenes* on stainless steel coupons using different media such as tryptic soy broth and brain heart infusion. The results agree with the work of Borucki, et al (2003) who found no significant differences ($p > 0.05$) between three *L. monocytogenes* isolates (15C18, 1159 and 2492) from different serotypes (1/2b, 4b) in their biofilm formation on stainless steel coupons. In chapter five, there was no significant difference ($p > 0.05$) in *L. monocytogenes* attachment to soil and hydroponically grown lettuce (Kyere *et al*., 2019). The initial attachment of *L. monocytogenes* was attributed to its movement via the formation of actin-based filaments on the flagella (Takeuchi et al., 2000). This might be the reason for the similarity in biofilm formation for all strains.

Contrary to this results, Chae & Schraft (2000) reported that there were significant differences ($p < 0.05$) in the attachment and biofilm formation of different *L. monocytogenes* strains to glass surfaces. Another study by Henriques & Fraqueza (2017) investigated the biofilm-forming ability of 113 *L. monocytogenes* strains on food contact surfaces. They reported that the biofilm formation of *L. monocytogenes* serogroups IIc and IVb were significantly higher than other serogroups studied. The strain specific mechanism of attachment of the different *L. monocytogenes* strains to the food contact surfaces might be the reason for their varying biofilm forming ability although this was not specified in the study.

From the results, the biofilm formation of the *L. monocytogenes* strains increased with time at both 4 and 10°C (Fig 6.2). From the SEM images, there were other microorganisms in lettuce extract interspersed with *L. monocytogenes* indicating mixed species biofilms. A higher magnification (12 000x) of the SEM image in Fig 6.4 makes it more visible. The prevalence of mixed biofilms formed on lettuce leaf surfaces and other fresh produce has been shown by other authors (Olmez & Temur, 2010). Mixed species biofilms have been reported to affect the overall biofilm community and can lead to the development of other phenotypes (Elias &Banin, 2012). Further investigation into naturally occurring biofilms found in lettuce extract will be necessary since biofilms of bacteria such as *Pseudomonas* have been reported to be able to protect *L. monocytogenes* cells from disinfectants (Bourion & Cerf, 1996). Biofilm formation reduces the efficiency of cleaning and sanitizing agents in the processing plant. The results of the present study show the ability of lettuce extracts to support *L. monocytogenes* biofilm formation in a mixed biofilm on stainless steel coupons at temperatures used in the fresh produce processing plants. This is a potential food safety hazard and may explain recent fresh produce related foodborne outbreaks originating from the processing environment.

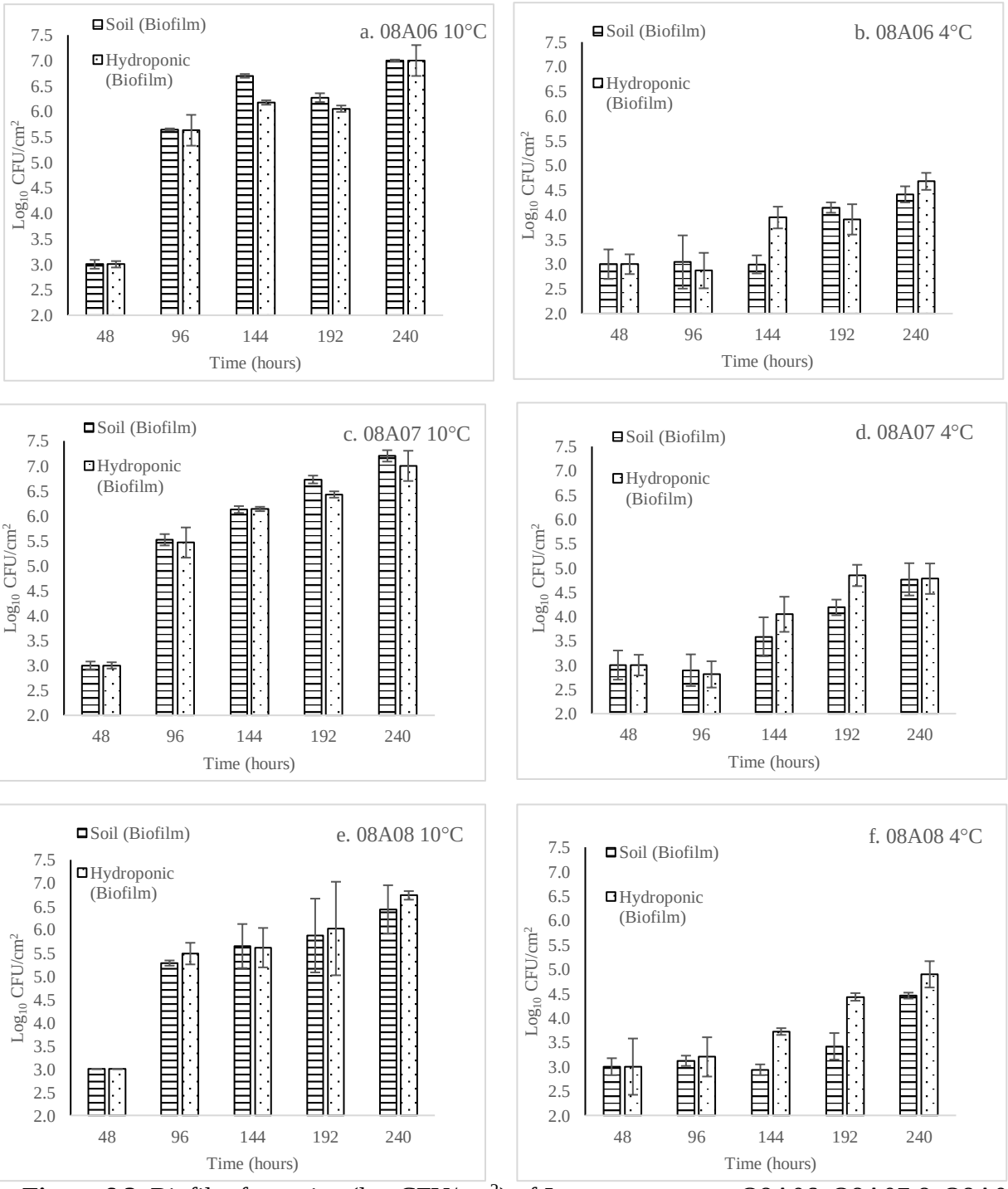


Figure 6.2. Biofilm formation (log CFU/cm^2) of *L. monocytogenes* O8A06, O8A07 & O8A08 on stainless steel coupons in soil & hydroponically grown lettuce extracts at 4°C & 10°C represented by the mean of three biological repeats with standard deviation error bars.

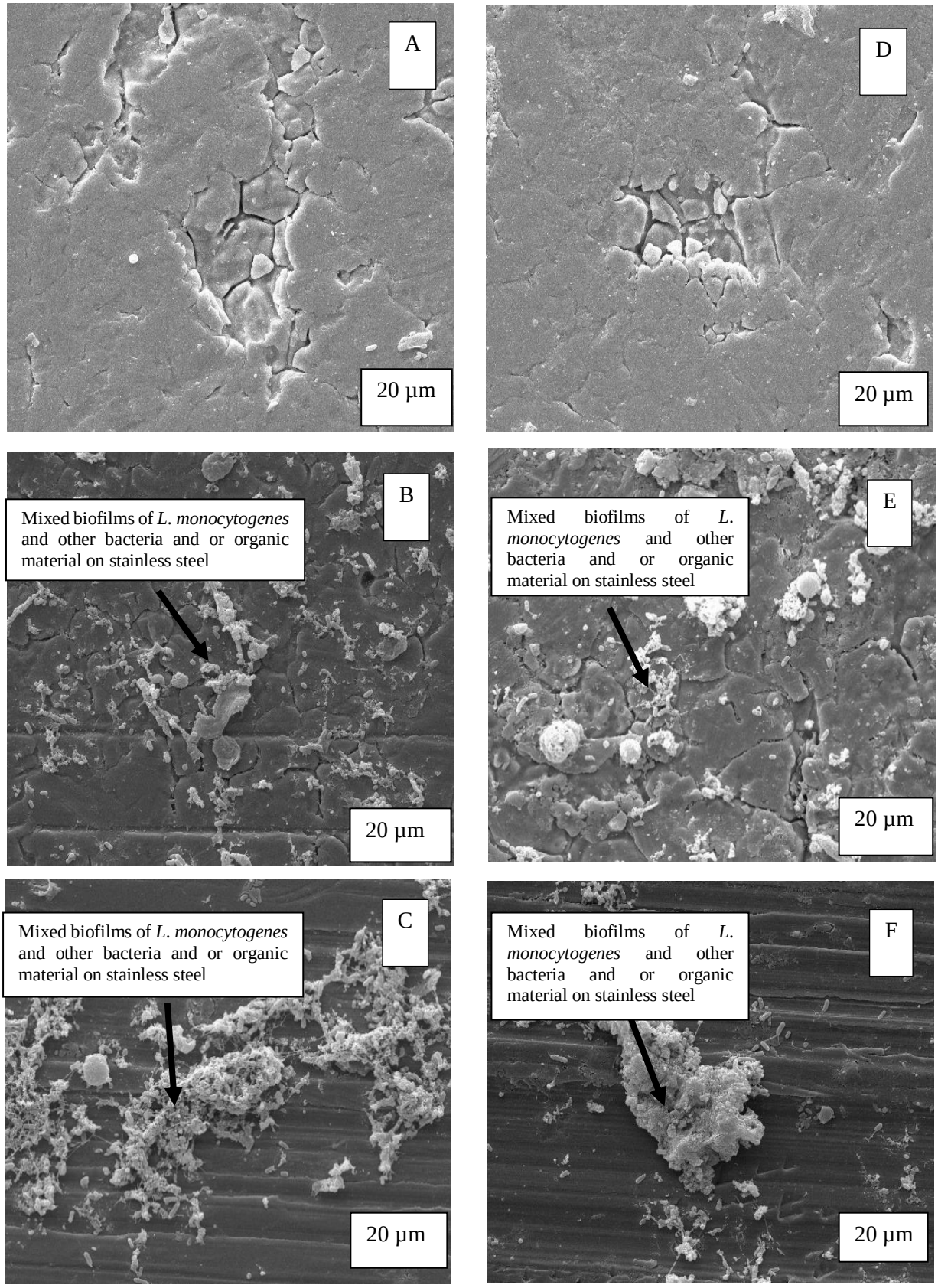


Figure 6.3. SEM images of *L. monocytogenes* biofilm formation on stainless steel coupons with soil and hydroponic lettuce extract at different times (t). A: t=0 (soil extract), B: t=96 h (soil extract), C: t=240 h (soil extract), D: t=0 (hydroponic extract, E: t=96 h (hydroponic extract) and F: t=240 h (hydroponic extract). All images (A-F) were taken with an accelerating voltage of 20 kV. Images from A to F were taken under 3 000 x magnification.

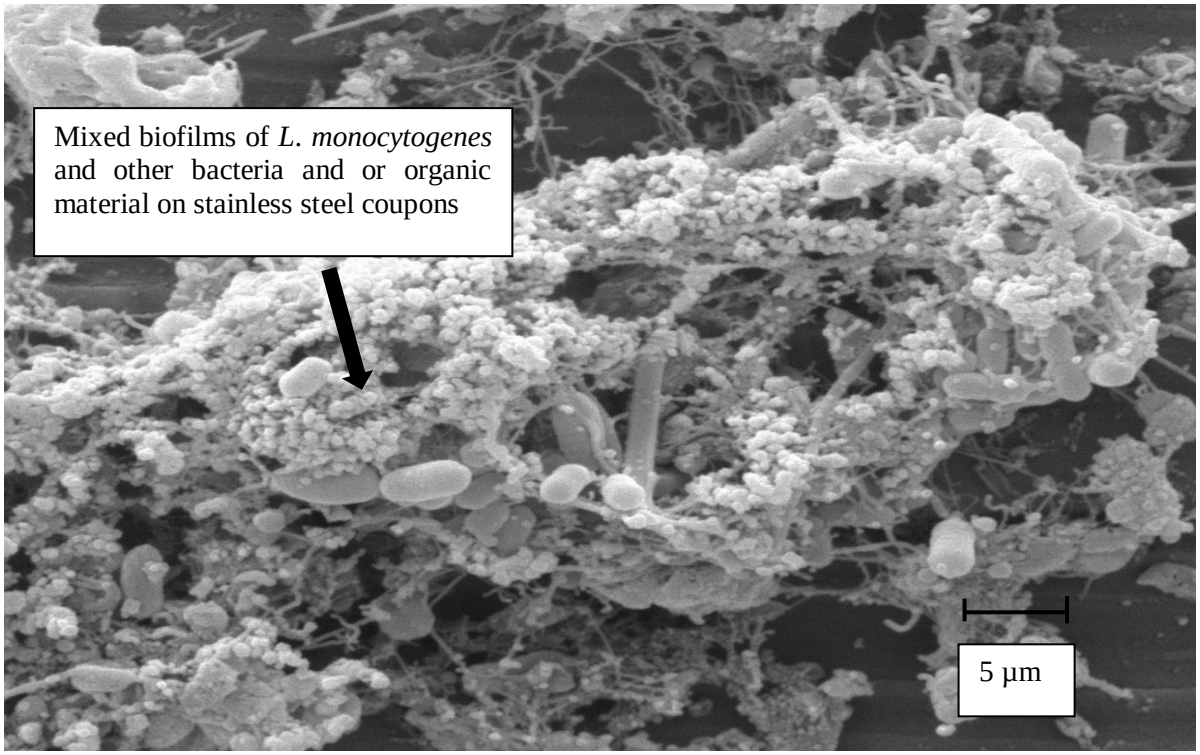


Figure 6.4. SEM images of *L. monocytogenes* biofilm formation on stainless steel coupons with hydroponic lettuce extract at t=240 h. Image was taken with an accelerating voltage of 20 kV and under 12 000 x magnification.

6.4 Conclusion

In this study, the ability of three different *L. monocytogenes* isolates isolated from fresh produce, to grow and form biofilms in a mixed biofilm on stainless steel in both soil and hydroponic grown lettuce extracts was confirmed. The results of this study demonstrate that control and prevention methods for *L. monocytogenes* should not only focus on lettuce leaf surfaces but also consider the potential biofilms that can be formed on processing plant surfaces exposed to the juice from lettuce leaves.

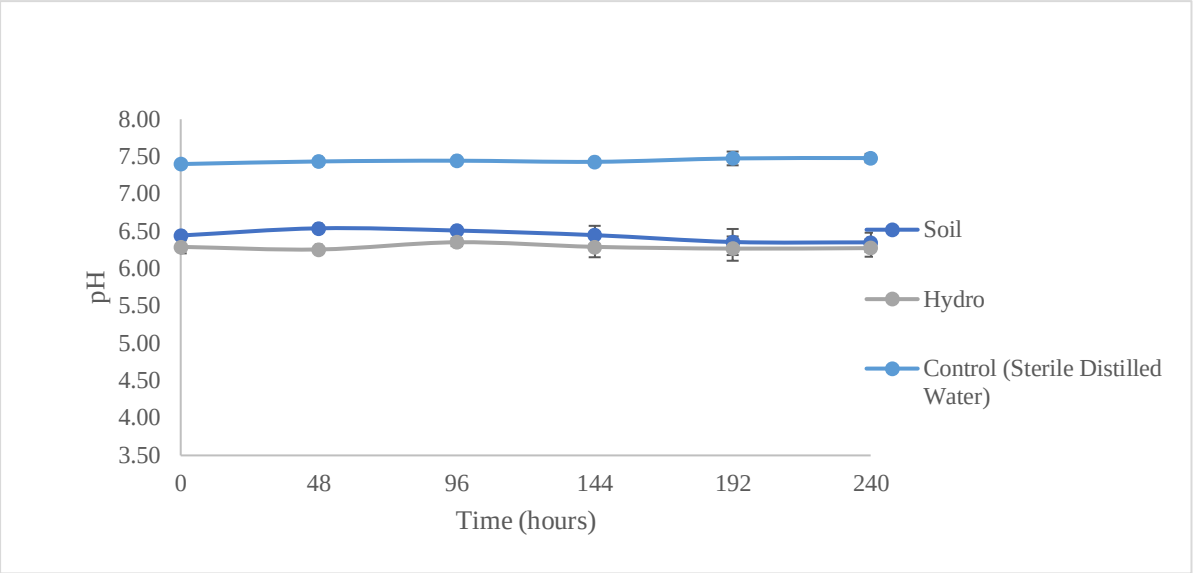
6.5 Summary of the chapter and the link to the next chapter

This chapter has revealed important information that will be useful for the fresh produce industry. The ability of lettuce extracts to support the biofilm formation of *L. monocytogenes*

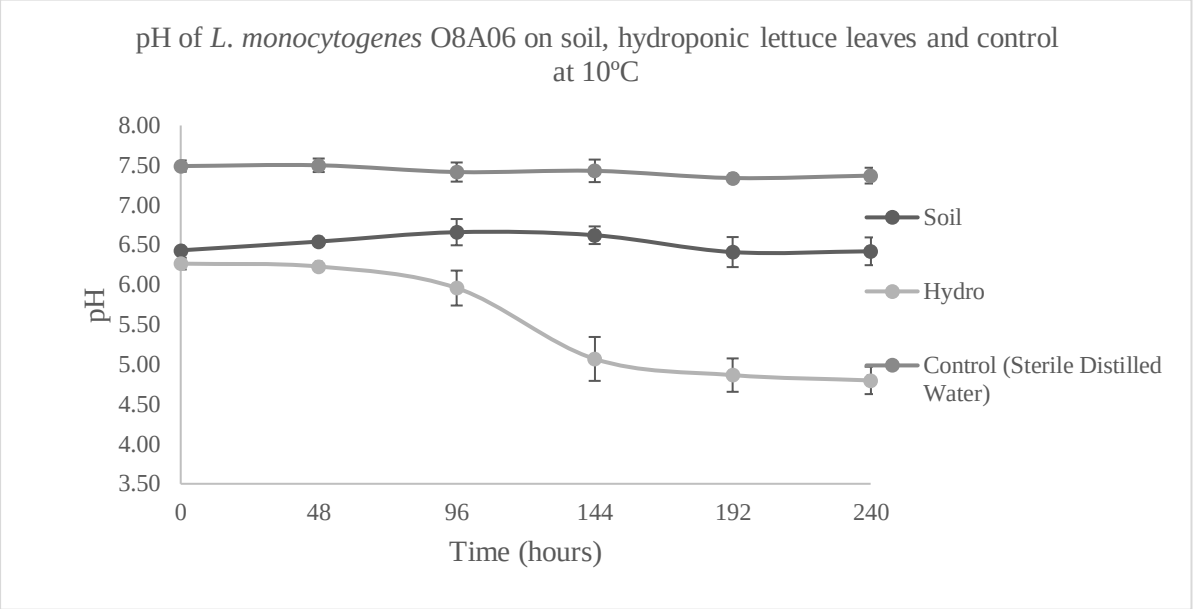
on stainless steel surfaces confirms the risk of biofilms as a source of contamination of product during processing. *L. monocytogenes* attachment, survival and growth on stainless steel showed no significant difference ($p > 0.05$) in the juice from hydroponic grown or soil grown lettuces. *L. monocytogenes* grows better in lettuce juices or extracts than on leaf surfaces. This might be due to the variations in the environment on the leaf surface (Hallman et al., 2001). The readily available nutrient source in lettuce juice as well as the high water activity of the medium might contribute to the better growth in lettuce juices than on the leaf.

Lettuce leaf surfaces do support *L. monocytogenes* biofilm growth and this is likely to be the original source of contamination entering a processing plant where biofilm may form and act as another source of contamination. One approach to controlling the source of contamination may be to treat the lettuce to reduce contamination. The next chapter covers the effect of UV stress as, a novel approach to reduce *L. monocytogenes* colonisation of lettuce leaf surfaces.

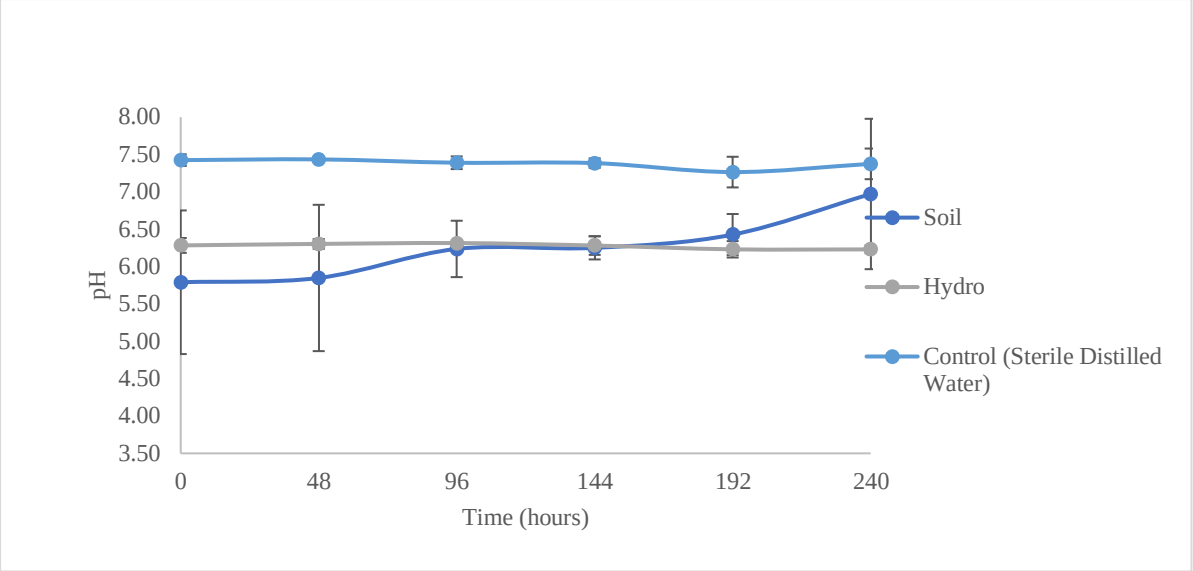
6.6 Supplementary Information



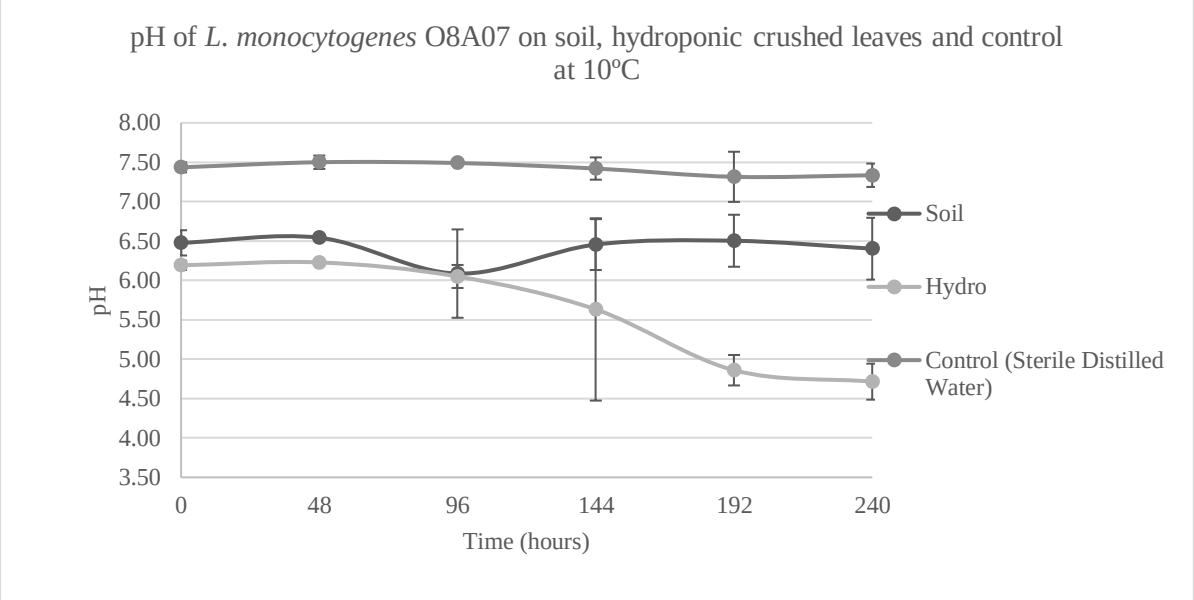
Supplementary Information S6.1. pH of lettuce juice after *L. monocytogenes* O8A06 has been added at 4°C for t = 0 to t = 240 h.



Supplementary Information S6.2. pH of lettuce juice after *L. monocytogenes* O8A06 has been added at 10°C for t = 0 to t = 240 h.



Supplementary Information S6.3. pH of lettuce juice after *L. monocytogenes* O8A07 has been added at 4 °C for t = 0 to t = 240 h.



Supplementary Information S6.4. pH of lettuce juice after *L. monocytogenes* O8A07 has been added at 10°C for t = 0 to t = 240 h.

6.7 References

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CHAPTER 7 Reduction of the attachment, survival and growth of *L. monocytogenes* on lettuce leaves by UV-C stress

Submitted to *Food Research International*

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Abstract

Mild stress of leafy greens by UV-C radiation has been reported to stimulate plant defences capable of reducing pathogens on produce surfaces. In this study, the attachment, survival and growth of *L. monocytogenes* was investigated on lettuces stressed with mild UV-C radiation (1.3 and 2.6 kJm^{-2}). Attachment of *L. monocytogenes* to UV-C stressed (1.3 kJm^{-2}) lettuce leaves after 1 h was reduced by 1.4-1.5 log CFU/cm^2. UV-C stress also reduced the numbers of *L. monocytogenes* on lettuce by 1.8-1.9 log CFU/g 96 h after inoculation, however a higher dosage of UV-C stress (2.6 kJm^{-2}) did not inhibit the survival of *L. monocytogenes*. The total phenolic compounds in lettuce increased following UV-C stress (1.3 kJm^{-2}) indicating the accumulation of polyphenols might have contributed to the inhibition of *L. monocytogenes* attachment and growth. Appropriate treatment with mild UV-C stress of lettuce can reduce the attachment, survival and growth of *L. monocytogenes* in lettuce and can therefore be explored further for commercial application in helping to improve fresh produce safety.

7.1 Introduction

L. monocytogenes is a Gram-positive bacterium which has been implicated in many fresh produce related outbreaks of food borne illness (Self et al., 2019). Fresh produce related outbreaks of foodborne illness as well as recalls due to pathogenic bacteria such as *L. monocytogenes* continue to be a concern in food safety (New Zealand Food Safety, 2020). There have been nine major fresh produce recalls associated with *L. monocytogenes* (from January to August 2020) in the US (United States Food and Drug Administration, 2020) Within the same time period, four major recalls of fresh produce due to *L. monocytogenes* were reported in New Zealand (NZ) (New Zealand Food Safety, 2020). In the previous chapters, the attachment of *L. monocytogenes* to lettuce surfaces as well as lettuce juice supporting the biofilm formation of *L. monocytogenes* on stainless steel coupons has been demonstrated (Kyere et al., 2019; Kyere et al., 2020). In addition, 5% of bagged lettuces sold in supermarkets in a major city in NZ were found to be contaminated with *L. monocytogenes* (Kyere et al., 2020). These recalls as well as contamination of lettuce with *L. monocytogenes* in retail outlets supports the need for research to investigate innovative control methods capable of reducing the risk from pathogens in fresh produce. Novel control methods are needed in the fresh produce industry as heat treatment, used for many other foods, is not suitable for these products and chemical treatments have limitations (Jung et al., 2014).

UV radiation (UV-A; 315-400 nm, UV-B; 280-315 nm and UV-C; 200-280 nm) has been used to inactivate microorganisms in the food industry, particularly in liquid foods and juices (Gómez-López et al., 2012). The application of UV-A and UV-B requires long exposure of food to radiation lasting several days to weeks (Aarrouf & Urban, 2020). The use of UV-C at high energy for a relatively short period of time has been explored as an effective treatment of food to reduce microbial load (Koutchma et al., 2016). The bactericidal effect of UV-C

radiation on fresh produce has been reported (Aarrouf & Urban, 2020; Collazo et al., 2019; Srey et al., 2014). UV-C treatment (390 mJ/cm^2) reduced pre-existing biofilm on *L. monocytogenes* on lettuce leaves by more than 3 log CFU/cm^2 (Srey et al., 2014). UV treatment (30 mW/cm^2) reduced *Salmonella enterica* on lettuce by 1.98 log CFU/cm^2 (Lippman et al., 2020). Direct application of UV radiation used to reduce pathogens already growing on leaf surfaces has sometimes been found to have a damaging effect on leaf quality (Tellez et al., 2016) and can affect affect the sensory quality of leaves (Tomás-Callejas et al., 2012). An alternative approach that minimises the damaging effect of UV radiation on leaves is mild UV stress of the leaf to generate an antimicrobial response in the plant (Urban et al., 2018). There have been several studies using UV-C stress to stimulate plant defence (Martínez-Sánchez et al., 2019; Urban et al., 2018; Vàsquez et al., 2017). In response to UV-C stress, plants produce phytochemicals and other phenolic compounds as a protective mechanism (Gamage et al., 2014). Some of these phytochemicals such as ascorbic acid and gallic acid found in leafy greens have antibacterial properties (Kang et al., 2018; Zudina et al., 2018). Other phytochemicals are beneficial to leafy greens by preventing plant diseases, strengthening plant structures such as the cell wall, enabling plants to withstand unfavourable climatic conditions and increasing shelf life of produce (Urban et al., 2016).

Lettuce stressed with a UV-C radiation dose of 0.85 kJ m^{-2} increased the resistance of lettuce to *Botrytis cinerea* L. (grey mould), a fungal pathogen (Vàsquez et al., 2017). UV-C stress (1.5 kJ m^{-2}) reduced mesophilic bacterial growth on spinach by 0.5-2 log CFU/g (Martínez-Sánchez et al., 2019). UV-C stress (2.6 and 5.2 kJ m^{-2}) increased some phytochemicals in broccoli such as sulforaphane nitrile which were capable of inhibiting *L. monocytogenes* growth (Gamage, 2015). These reports suggest the potential for UV stress to be used in controlling pathogenic bacteria on leafy greens. However, no studies have specifically investigated the effects of UV-C stress of lettuce on *L. monocytogenes*.

This is the first report of the effect of UV-C stress of pre-harvest lettuce on *L. monocytogenes* attachment, survival and growth. The importance of optimising the dose to avoid damage to the leaf while generating an antimicrobial stress response was also shown.

7.2 Materials and Methods:

7.2.1 Growth of lettuce

Lettuce plants were grown in the Plant Growth Unit of Massey University, Palmerston North, New Zealand. Buttercrunch lettuce seeds (*Lactuca sativa* L. *var. capitata*) were grown under the same conditions as described previously (Kyere et al., 2019). Lettuce plants were grown for four weeks in a greenhouse at an average temperature of 20 °C. The soil grown lettuce seeds were sown in potting mix in thin rows at a depth of 5 mm with 35 cm between each row (Sanguandeekul, 1999).

7.2.2 UV-C treatment

UV-C treatment was carried out with the UV equipment previously described by (Gamage et al., 2014). The UV equipment consists of four germicidal UV-C lamps (Philips ultraviolet TUV 30W/G30T8, Holland) with a wavelength of 253.7 nm. Whole lettuce plants rooted in pots of potting mix were placed under the UV lamps at 50 cm from the lamps. The UV light intensity was measured with an ILT1400 radiometer photometer (International Light Technologies, USA). The duration of UV radiation exposure to the lettuce leaves of two and five minutes corresponded to doses of 1.3 kJm^{-2} and 2.6 kJm^{-2} respectively. The UV-C lamps were turned on for at least 30 min before exposure to lettuce leaves and briefly turned off while putting leaves in the chamber. All lettuce plants were stressed with UV after sunset. Non-UV stressed plants were used as controls. Three independent experiments each with three lettuce plants

were conducted. Scanning electron microscopy (SEM) images of both lettuce types (UV stress and non-UV stress) were taken after UV treatment to check if the UV dose used had any negative effect on leaf surface structures. After UV stress, all plants were kept in the greenhouse with the same growth conditions described above.

7.2.3 Bacterial Strains and Inoculum Preparation

The inoculum preparation was prepared as previously described (Kyere et al., 2020). *L. monocytogenes* O8A07 and O8A08 (cabbage isolates) from the New Zealand Institute for Plant and Food Research Limited (PFR) culture collection were maintained at -80 °C on protective beads with Brain Heart Infusion (BHI) Broth (Becton, Dickinson & Company, Le Pont de Claix, France) and 20 % (v/v) glycerol. Frozen cultures were first transferred to 9 ml BHI broth at 30 °C for 12 – 14 h with agitation at 120 rpm (Gallery Orbitron Shaker, INFORS HT, Germany) and then sub-cultured in BHI broth for an additional 12 – 14 h at 30 °C before use.

L. monocytogenes isolates were centrifuged at 4400 g for 10 min at room temperature (Eppendorf Centrifuge 5702, Hamburg, Germany). Phosphate Buffered Saline (PBS, Code OPM343, Fort Richard Laboratories, Auckland) was used to wash the cell pellet and then resuspend in 0.1 % sterile Buffered Peptone Water (BPW, GranuCult™, Merck KGaA, Billerica MA, USA). Serial dilutions were made in BPW to obtain a final cell count of approximately 10^5 CFU/ml which was used as the initial inoculum.

7.2.4 Inoculation of lettuce for attachment

Lettuce leaves were collected 24 h after UV stress. The attachment assay used previously (Kyere et al., 2019) was followed. Leaves were cut into 2 cm by 2 cm squares with a sterile cutter. Leaves were rinsed in sterile distilled water and placed under a laminar flow cabinet for 30 min to dry. The 2 cm by 2 cm leaves were inoculated with a 500 µl inoculum to cover the

whole surface. The *L. monocytogenes* inoculum was left on the leaf surface for 5 min, 30 min and 60 min for attachment. After the attachment exposure times, lettuce leaves were washed with 2 ml sterile distilled water to remove unattached cells. SEM images of *L. monocytogenes* attached to both UV and non-UV stressed lettuce were taken after every attachment exposure time.

7.2.5 Inoculation of lettuce for survival and growth studies

Lettuce leaves were collected for *L. monocytogenes* survival and growth studies 24 h after UV stress. Lettuce leaves were sprayed with a 5 log CFU/ml *L. monocytogenes* inoculum. For spray inoculation, a 500 ml LabServ sprayer (Thermo Fisher Scientific, New Zealand) was used three times to spray lettuce leaves in a horizontal position 6 cm above the base in a biosafety cabinet. Initial investigations in our laboratory revealed that approximately 1.5 ml of inoculum was applied to each lettuce plant by spraying for 2 s. Leaves were dried in a laminar flow cabinet for 60 min. After drying, 10 g of lettuce leaves from plants growing in five different pots were tested for the initial number of *L. monocytogenes* attached. Both control (non-UV) and UV stressed lettuce leaves with the *L. monocytogenes* inoculum were transferred to a 90 % relative humidity chamber at 20°C. *L. monocytogenes* survival and growth on lettuce plants were enumerated after every 24 h for four days.

7.2.6 Enumeration of *L. monocytogenes* to monitor attachment, survival and growth on lettuce leaf surfaces

Enumeration for *L. monocytogenes* attachment followed the procedure described previosuly (Kyere et al., 2019). Leaf squares (2 cm by 2 cm) were aseptically placed into 9 ml 0.1% sterile peptone water filled with 10 g of sterile glass beads. *L. monocytogenes* cells were released from lettuce leaf surfaces, mixing by vortex for 2 min. Serial dilutions were plated on Palcam agar and plates were incubated for 48 h at 30 °C.

For the enumeration of *L. monocytogenes* survival and growth on lettuce leaves, the method described previously (Kyere et al., 2020) was used. Ten g of lettuce from each pot were homogenised with 90 ml of 0.1 % peptone water (GranuCult®, Merck, KGaA, Germany) using a Stomacher Lab Blender (AES-Chemunix) for 120 s at a speed of 250 rpm. Serial dilutions of the homogenates were plated on Palcam agar. Plates were incubated for 48 h at 30 °C.

7.2.7 Scanning electron microscopy

The attachment of *L. monocytogenes* on UV stressed and non-UV stressed lettuce leaves was confirmed by SEM. SEM images of both UV stressed and non-UV stressed lettuce leaves were observed before they were inoculated with *L. monocytogenes*. The attachment of *L. monocytogenes* on UV stressed and non-UV stressed lettuce was also observed after every exposure time. Lettuce leaves were sent to the Manawatu Microscopy and Imaging Centre, Massey University, Palmerston North for imaging at each sampling time. Lettuce leaves were prepared for SEM as described previously (Kyere et al., 2020), and viewed under the FEI Quanta 200 Environmental scanning electron microscope (FEI Company, Hillsboro, OR) at 20 kV.

7.2.8 Determination of total phenolic compounds

Total phenolic content was determined using the modified Folin-Ciocalteu reagent method (Oh et al., 2009). Ten g of fresh lettuce leaves were homogenised in 60 mL 80% (v/v) acetone with an homogeniser (Breville CG2B, Australia) for 2 min. The homogenate was incubated in darkness at 4°C overnight. It was then centrifuged at 3000 g for 2 min with an Eppendorf centrifuge (5702 Eppendorf AG Hamburg, Germany). 270 µL H_2O, 1.5 mL 1/10 dilution Folin-Ciocalteu reagent (Sigma-Aldrich, USA) and 1.2 mL 7.5% (w/v) Na_2CO_3 was added to 100 µL of the homogenate. The homogenate was mixed by vortex for 30 seconds and incubated in a

water bath at 45°C for 15 min. Absorbance was read at 760 nm and total phenols were quantified using a freshly made gallic acid standard curve. Each sample (UV stressed and non-UV stressed lettuce leaves) was tested in triplicate.

7.2.9 Statistical analysis

All experiments were done with three biological repeats with each comprising three technical repeats. An analysis of variance (ANOVA) was performed at a significance level of $p < 0.05$ using Minitab Statistical Software (Minitab version 17, State College, Pennsylvania, USA) and Tukey's test was applied to determine significant differences. The numbers of *L. monocytogenes* cells attached to the leaves were converted to log CFU/cm^2 and the numbers of *L. monocytogenes* cells recovered from lettuce leaves were converted to log CFU/g.

7.3 Results and discussion

7.3.1 The effect of UV-C stress on *L. monocytogenes* attachment, survival and growth on lettuce surface

The attachment of *L. monocytogenes* (O8A07 and O8A08) to non-UV stressed lettuce was significantly higher ($p < 0.05$) than UV stressed lettuce at 30 and 60 min attachment (Fig. 7.1). There was no significant difference ($p > 0.05$) between the two strains in terms of their attachment to both non-UV stressed and UV stressed lettuce. *L. monocytogenes* attachment to non-UV stressed lettuce showed a time-dependent response with attachment increasing slowly with increasing exposure times. Exposure of lettuce to 5 log CFU/ml *L. monocytogenes* for 60 min resulted in an approximate 2.6 log CFU/cm² attachment. However, exposure to UV stressed lettuce to 5 log CFU/ml of *L. monocytogenes* for 60 min resulted in 1.1-1.2 log CFU/cm^2 attachment (Fig. 7.1) and did not increase with time. Attachment of both O8A07 and O8A08 *L. monocytogenes* strains was inhibited on UV stressed lettuce (Figs. 7.1 and 7.2).

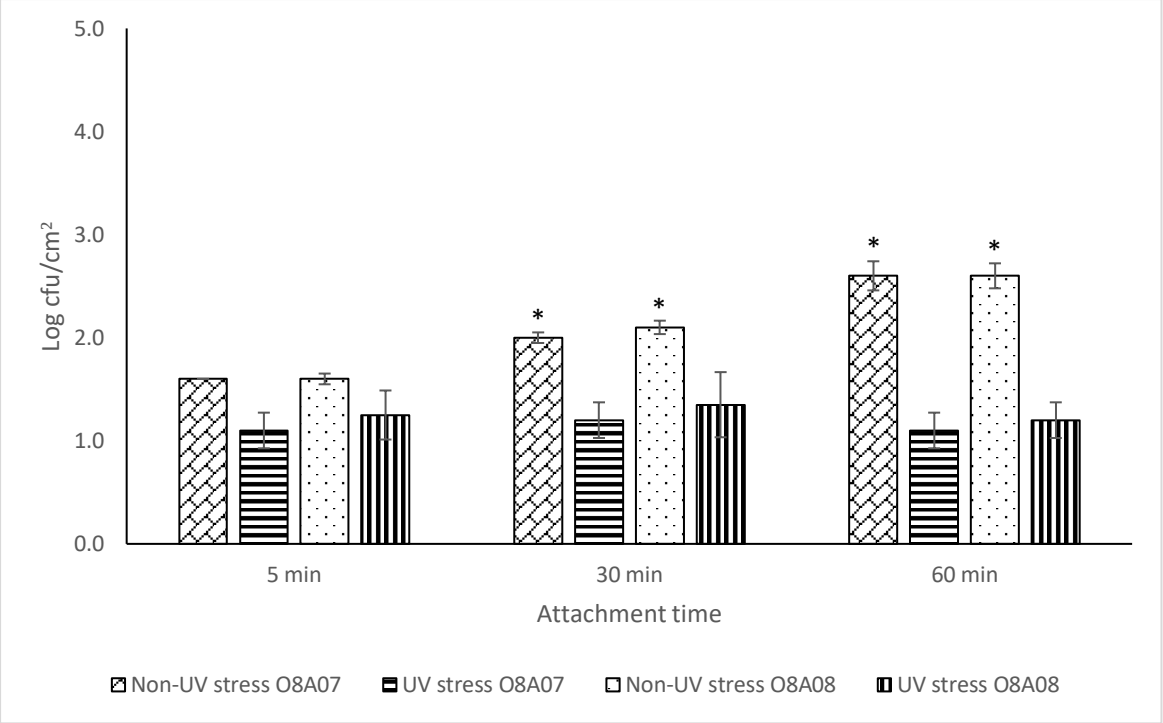


Figure 7.1. Effect of UV-C stress on *L. monocytogenes* (O8A07 and O8A08) attachment (log CFU/cm^2) to lettuce leaves at exposure times of 5 min, 30 min and 60 min. Data is represented by the mean of three biological repeats with standard deviation error bars. Statistical differences were determined with ANOVA and Tukey's test at $p < 0.05$. Bars with an asterisk (*) denote a significant difference.

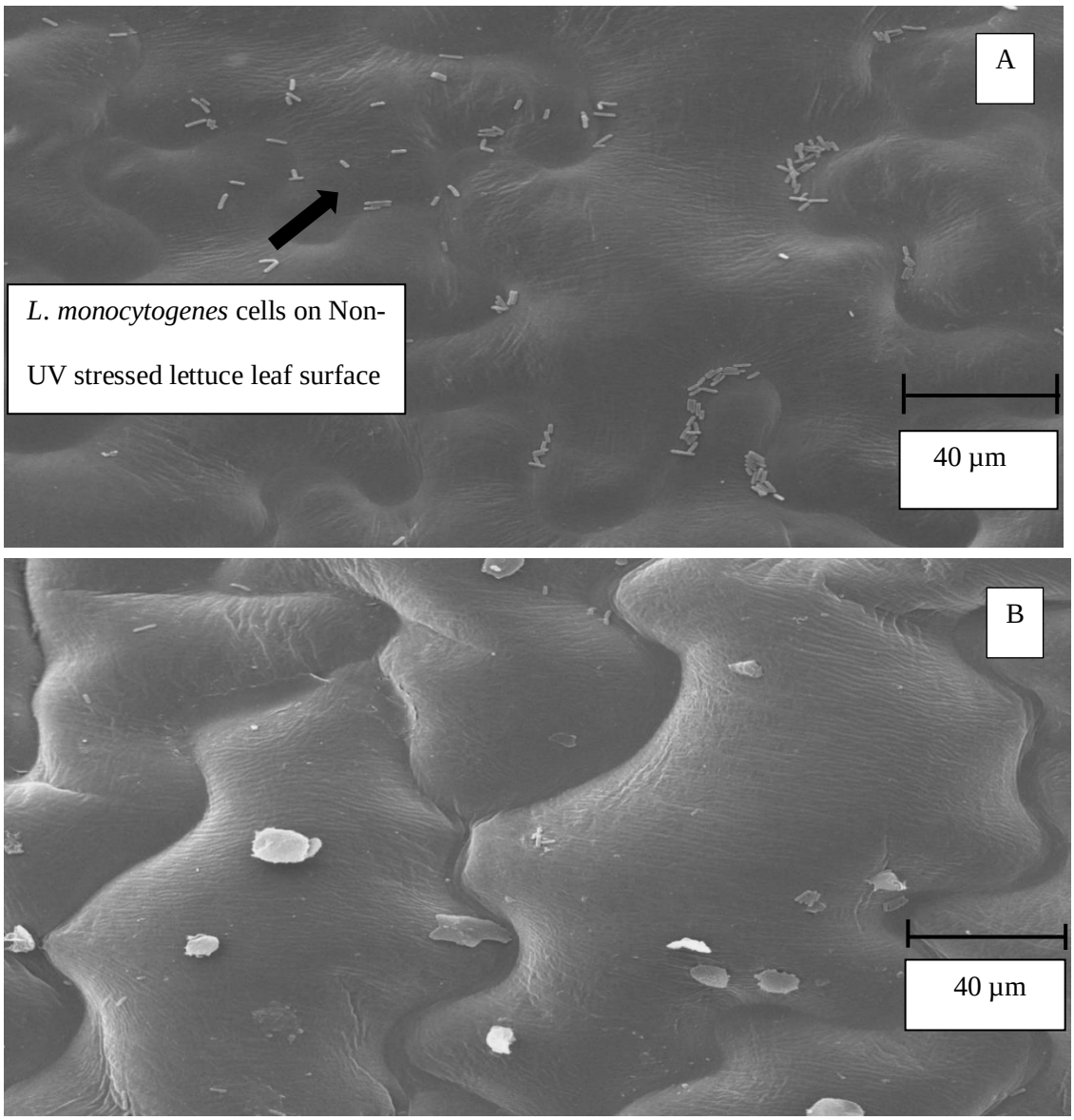


Figure 7.2. SEM images of *L. monocytogenes* attachment on Non-UV stressed and UV stressed lettuce leaves after 60 min exposure time. A: Non-UV stressed and B: UV stressed lettuce. Images were taken with 1500 x magnification and a scale of 40 µm.

L. monocytogenes survival and growth on non-UV stressed lettuce was also significantly higher ($p < 0.05$) than UV stressed lettuce after 24, 48 and 96 h (Fig. 7.3). After 60 min drying, approximately 3 log CFU/g of the *L. monocytogenes* survived on the non-UV stressed leaves whereas only 2 log CFU/g survived on the UV stressed leaves. *L. monocytogenes* numbers on non-UV stressed lettuce grew to about 4.6-4.7 log CFU/g after 96 h whereas *L. monocytogenes* on UV stressed lettuce only grew to 2.7-2.8 log CFU/g after 96 h. These results suggest that *L.*

monocytogenes is less likely to attach and grow on UV stressed lettuce than non-UV stressed lettuce.

SEM images of both non-UV and UV stress lettuce did not show any structural differences, which may suggest the dosage used did not influence leaf surface properties. However, a UV dosage of 2.6 kJm^{-2} resulted in lettuce leaves with brown spots 24 h after stressing (Supplementary Figure 7.1) and the growth of *L. monocytogenes* on lettuce stressed with 2.6 kJm^{-2} was not inhibited but was actually slightly increased after 96 h (Fig. 7.4). Rather than evoking a stress response, the higher dose of UV damaged the lettuce leaves. There is therefore a need to optimise the UV dose to achieve a protective stress response but avoid damage to the lettuce tissue that may increase the colonisation of lettuce by *L. monocytogenes*.

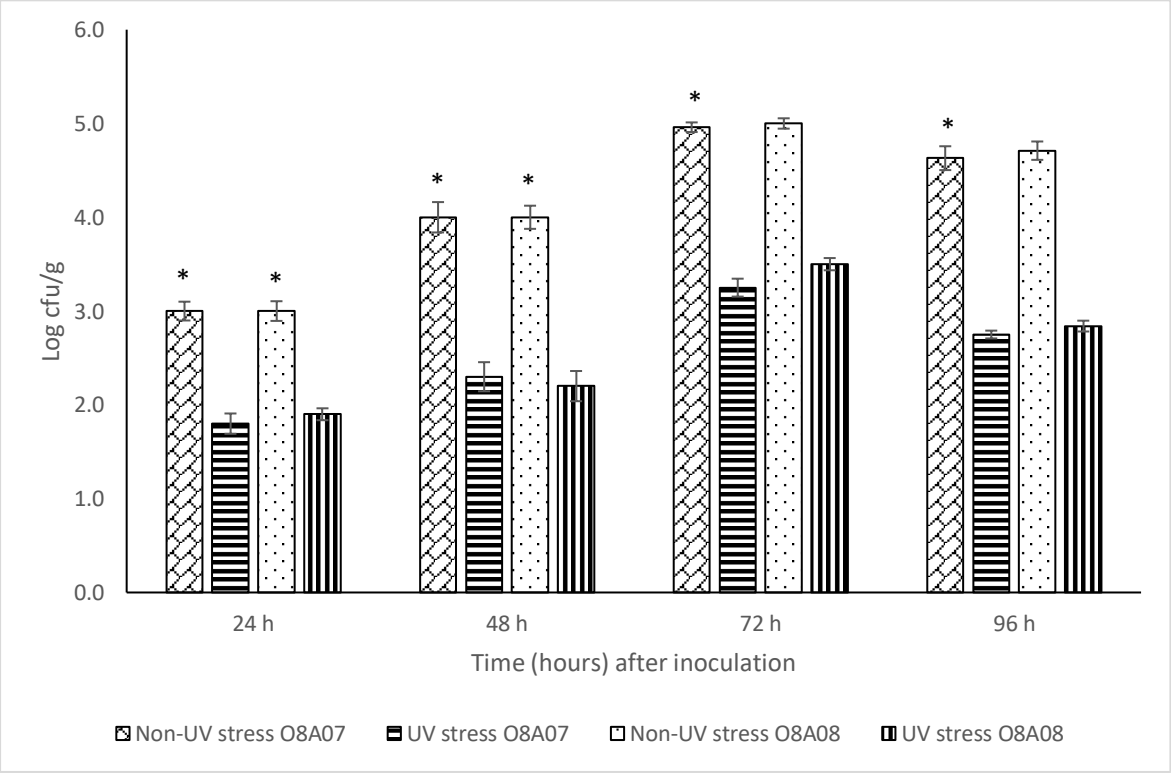


Figure 7.3. Effect of UV-C stress (1.3 kJm^{-2}) on *L. monocytogenes* (O8A07 and O8A08) survival and growth (log CFU/g) on lettuce leaves**.** Data is represented by the mean of three biological repeats with standard deviation error bars. Statistical differences were determined with ANOVA and Tukey's test at $p < 0.05$. Bars with an asterisk (*) denote a significant difference.

Most studies on the effect of UV stress on leafy greens have focused on plant fungal pathogens (Ouhibi et al., 2015; Vàsquez et al., 2017). UV-C (0.85 kJm^{-2}). Stress of lettuce leaves inhibited the growth of *Botrytis cinerea* (a fungal pathogen). UV-C stress resulted in 10-20% reductions in fungal lesions on lettuce surfaces by the 6^{th} and 7^{th} day after inoculation (Vàsquez et al., 2017). Similarly, Ouhibi et al. (2015) reported a significant inhibition in the growth of *Sclerotinia minor* (a fungal pathogen) by UV stress on lettuce by the 4^{th} day after inoculation.

There have been some studies on the effect of UV stress on the growth of human pathogenic bacteria on leafy greens. Gamage et al. (2014) reported that an aqueous extract from UV stressed (5.2 kJm^{-2}) broccoli significantly reduced growth of *L. monocytogenes* after 16 h. The relatively high UV dose (5.2 kJm^{-2}) that they used would have damaged our lettuce plants as shown in our results (Fig 7.4). Broccoli branchlets are structurally robust (Lopez-Sanchez et al., 2011) which may explain why the 5.2 kJm^{-2} dose did not have an adverse effect on broccoli plants.

Direct application of UV treatment on pathogenic bacteria in leafy greens inhibits bacterial growth by damaging bacterial DNA. This leads to the formation of dimers between pyrimidine residues in the DNA strands thereby halting DNA replication (Montgomery & Banerjee, 2015; Yin et al., 2013). The reduction in the number of *L. monocytogenes* (1.8-1.9 log) on lettuce by UV stress as shown by our results is similar to the reduction that has been achieved by some studies on the application of UV to plants already colonised by bacteria. For example, UV treatment (0.43 Jcm^{-2}) reduced the biofilm formation of *L. monocytogenes* on Romaine lettuce by 1.19 log CFU/ml after 24 h (Montgomery & Banerjee, 2015). The reductions observed in the present trials were higher than that observed by direct UV application to the bacteria on plant surface as reported by (Martínez-Hernández et al., 2015). A UV-C dose of 2.5 kJm^{-2} reduced *L. monocytogenes* on broccoli by only 0.72 log (Martínez-Hernández et al., 2015). This shows the need for further investigations into how UV-C radiation can be used to cause a

stress response in fresh produce as this can have a greater effect on reducing pathogen contamination without causing damage to leaf tissues.

The effect of mild UV stress on leafy greens to prevent pathogen colonisation has been reported to be influenced by several factors including leaf structures such as cuticles, stomata and epidermis (Fan et al., 2017). UV stress of leaves can change the leaf surface structure by damaging the epidermal covering (Vàsquez et al., 2017). Depending on the extent of damage, this can either be beneficial or harmful to pathogenic bacteria. For example, it was reported that UV stress (3.4 kJm^{-2}) weakened the epidermal protection of lettuce leaves resulting in a greater colonisation by *Botrytis cinerea* L. (a fungal pathogen). Similarly, a UV dosage of 24 kJm^{-2} damaged baby spinach tissues resulting in greater colonisation with *L. monocytogenes* (Escalona et al., 2010). On the other hand, Syamaladevi et al., (2015) found a UV dose of 1.03 kJm^{-2} reduced *Penicillin expansum* by 1.8 log on apple surfaces without affecting the epidermis. UV stress has also been found to affect leaf surface properties such as hydrophobicity and contact angle measurements (Syamaladevi et al., 2015). Greater UV doses are needed to achieve microbial reductions on produce with rough surfaces. For example, a greater UV dose was required to achieve similar reduction in *P. expansum* (a fungal pathogen) on strawberry as compared to apples (Syamaladevi et al., 2015). In addition to these factors mentioned above, other properties such as leaf surface microflora, leaf age and source of leaf may also be factors in the effects of UV stress (Urban et al., 2016).

Analysis of the total phenolic content of UV stressed and non-UV stressed lettuce leaves indicated a significant increase ($p < 0.05$) in the total phenolic content of UV stressed lettuce (Fig. 7.5). This indicates an accumulation of the total polyphenols in lettuce as a stress response. Although the exact mechanism for reduced attachment, survival and growth on UV stressed leaves is unknown, it is likely related to this increase in polyphenols. Other researchers

have also reported an increase in total polyphenol content in lettuce and other fresh produce after UV stress (Lee et al., 2014; Ouhibi et al., 2015; Tsormpatsidis et al., 2008). Some major polyphenols in lettuce that accumulate in response to UV stress include chlorogenic acid, chicoric acid, caffeic acid and quercetin-3-O-glycoside (Gamage et al., 2014; Wargent et al., 2015). Some of these polyphenols are bactericidal to foodborne pathogens (Lou et al., 2011). A study to investigate the antibacterial activity of chlorogenic acid on different foodborne pathogens; *Staphylococcus aureus*, *Bacillus subtilis* and *Salmonella* Typhimurium was conducted by (Lou et al., 2011). Chlorogenic acid effectively inhibited the growth of all tested pathogens with minimum inhibitory concentrations ranging from 20-80 μg/mL. Chlorogenic acid increases plasma membrane permeability leading to the breakdown of membrane function. In extreme cases, this leads to leakages in cellular cytoplasmic components (Lou et al., 2011). Caffeic acid has also been reported to have inhibitory effects on several foodborne pathogens (Wen et al., 2003; Zhang et al., 2020). A combination of caffeic acid (1.5 mg/mL) with fosfomycin (50 mg/L) significantly reduced the growth of *L. monocytogenes* with a minimum inhibitory concentration of 2.25 mg/L (Zhang et al., 2020). Inhibitory effects of caffeic acid against *L. monocytogenes* have also been reported in other studies (Pernin et al., 2019; Vallejo et al., 2020).

Quercetin has been found to reduce the attachment and biofilm formation of *L. monocytogenes*. 0.2 mM quercetin significantly ($p < 0.05$) reduced *L. monocytogenes* attachment to stainless steel coupons by 1.48 log CFU/cm^2. Also, a 0.2 mM concentration significantly reduced *L. monocytogenes* biofilm cells by 1.96 log CFU/cm^2. The ability of quercetin to inhibit *L. monocytogenes* attachment and biofilms was attributed to its ability to inhibit nucleic acid synthesis (Vazquez-Armenta et al., 2018).

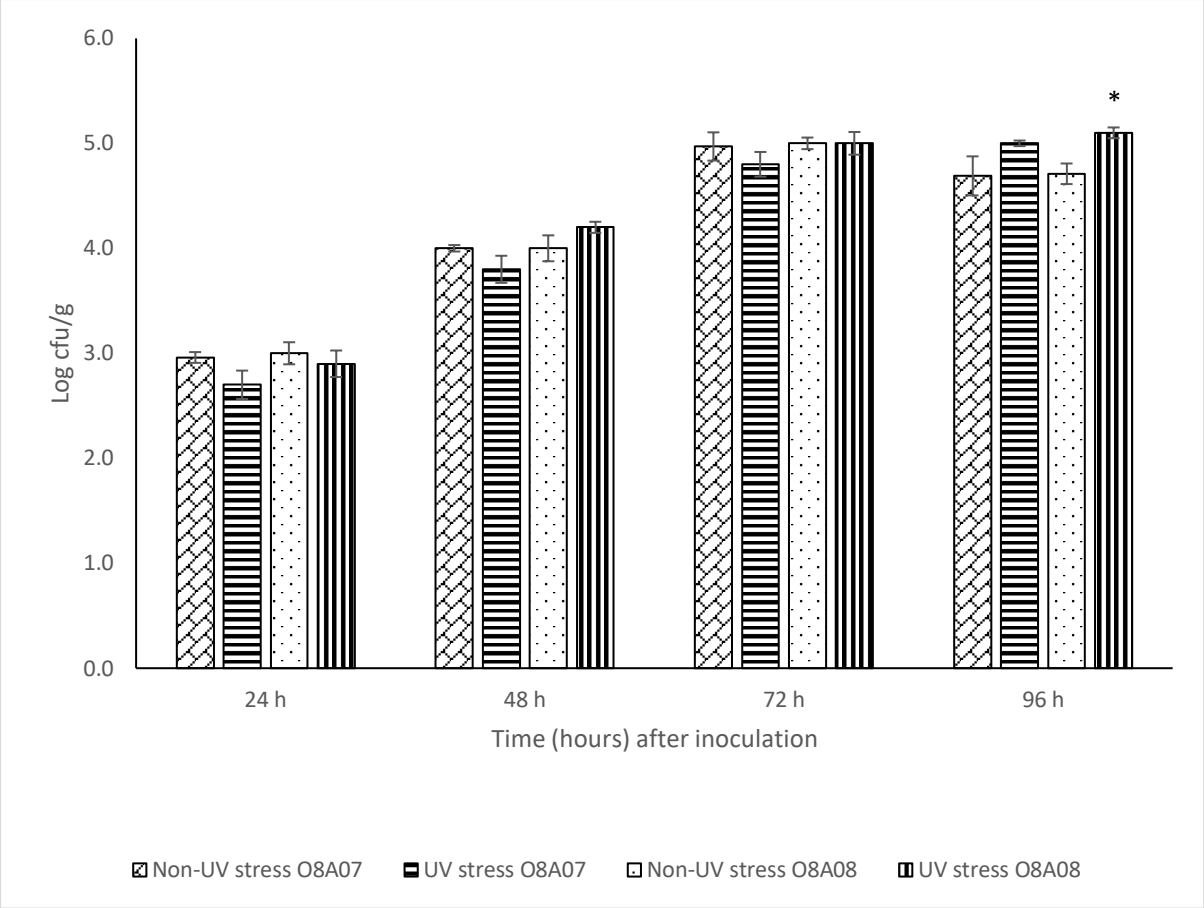
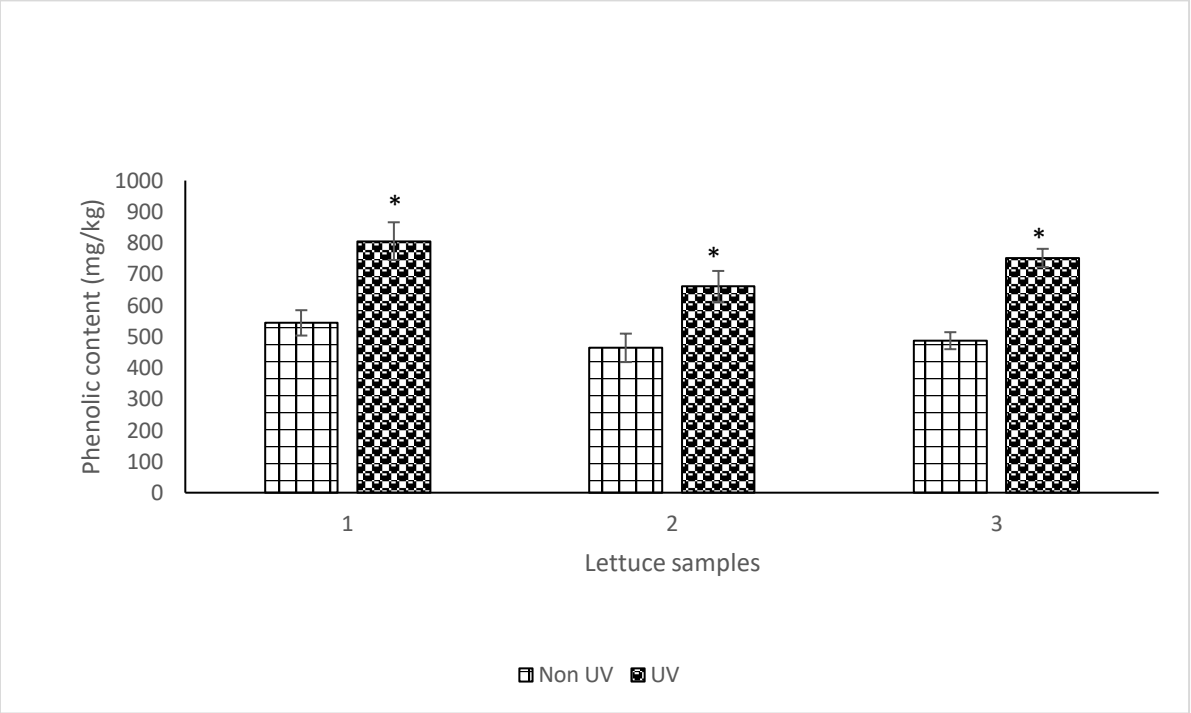


Figure 7.4. Effect of UV-C stress (2.6 kJm^{-2}) on *L. monocytogenes* survival and growth on lettuce leaves. Data is represented by the mean of three biological repeats with standard deviation error bars. Statistical differences were determined with ANOVA and Tukey's test at $p<0.05$. Bars with an asterisk (*) denote a significant difference.

Figure 7.5. Effect of UV-C stress (1.3 kJm^{-2}) on total phenolic content in lettuce leaves.



Data is represented by mean of three biological repeats with standard deviation error bars. Statistical differences were performed with ANOVA and Tukey's test at $p < 0.05$. Bars with an asterisk (*) denote UV-C stressed lettuce with significantly higher polyphenols than non-UV stressed lettuce.

7.4 Conclusion

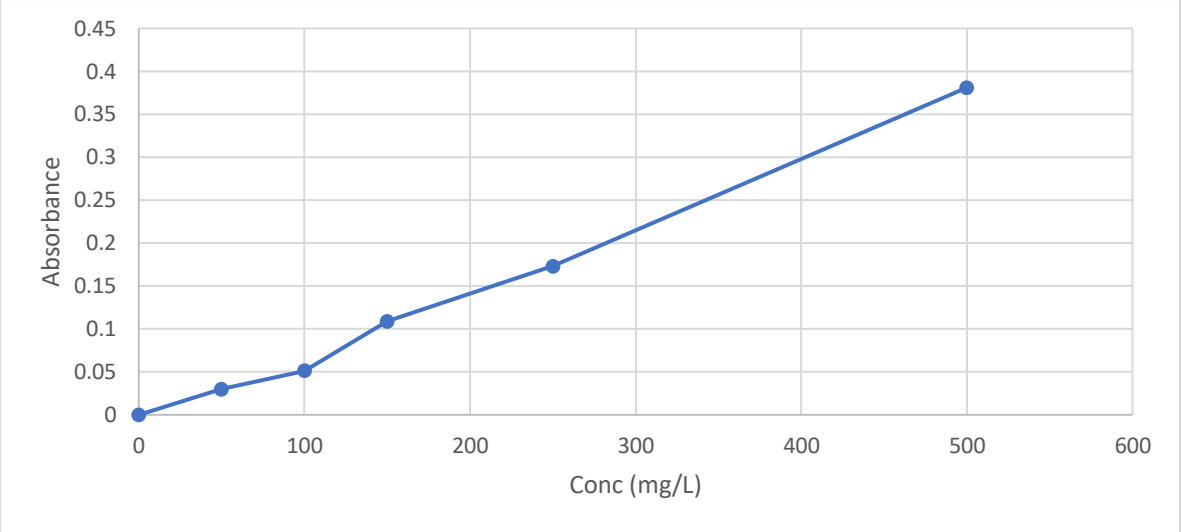
I report for the first time the ability of UV-C stress to reduce the attachment and growth of *L. monocytogenes* on lettuce leaves. Total polyphenol compounds in UV stressed lettuce increased compared to the non-UV stressed lettuce. Some of the polyphenols are known to inhibit *L. monocytogenes* growth which might explain the observed reductions. The application of UV-C should be further explored as a potential pathogen control in the fresh produce industry. Exposure of preharvest lettuce in the field to UV-C radiation at the right dosage could significantly ($p < 0.05$) reduce the attachment and growth of *L. monocytogenes*. Further studies to investigate the effect of UV-C stress on the gene expression of lettuce genes and the effect of these genes on *L. monocytogenes* growth will be worth investigating. The results from this

study indicate the potential benefit of the pre-harvest treatment of lettuce with UV-C to generate a stress response (hormesis) in the lettuce to reduce contamination with *L. monocytogenes* and improve food safety.

7.5 Supplementary information

Supplementary Figure S7.1. UV-C (2.6 kJm-2) stress of lettuce leaf elicit brown spots on leaf surface 24 h after UV stress.

Supplementary Figure S7.2. Lettuce leaves under UV-C bulbs in a UV equipment.



Supplementary figure S7.3. Total phenolic content calibration curve with gallic acid

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CHAPTER 8 Summarising discussion and conclusions

8.1 Introduction

The control of pathogenic bacteria on fresh produce during pre-harvest, postharvest and at retail sale is important for two reasons. Firstly, there are many points in the fresh produce production chain (from farm to fork) where contamination can occur (Jiang et al., 2004). This is evident in many cases of pathogen contamination that have been reported after sanitization or washing of produce (Kyere et al., 2020). Bacterial inactivation by high temperatures, one of the most reliable means for inactivating pathogens, is not available during processing due to the damage it will do to the fresh produce (Jung et al., 2014).

A survey of *Listeria* contamination in lettuces sold at retail confirmed the existence of the problem concerning safety of fresh produce (Kyere et al., 2020). Lettuces sold in supermarkets were contaminated with relatively high numbers (2-2.5 log CFU/g) of *L. monocytogenes* and *Listeria* spp. All the lettuces that were confirmed positive were in bags as against being sold loose (Kyere et al., 2020). This survey justified the need for the fresh produce industry to better ensure the production of safe salad products. The results of the survey also suggest that novel control methods capable of reducing *L. monocytogenes* on lettuce are important to be investigated. It is generally assumed that, bagged salads have a lower risk of microbial contamination since bagging is done to prevent contamination (Olaimat & Holley, 2012). However, this survey showed that bagged salads pose a higher food safety risk for listeriosis (Kyere et al., 2020). This calls for the produce industry to optimise packaging conditions for

bagged salads, minimising pathogen contamination and growth as well as maintaining the quality of salads.

In chapter four, a comparison was reported comparing hydroponic grown and soil grown lettuce with regard to their nutrient content and bacterial composition. Hydroponic grown lettuce is grown in a controlled environment and has been associated with a lower risk of microbial contamination (Selma et al., 2012). Unlike soil grown lettuce, hydroponic grown lettuces are grown in nutrient solutions with optimized growth conditions (Miller, 2019). The soil contains many bacteria and may include opportunistic pathogens (Alegbeleye et al., 2018). It was hypothesized that the use of hydroponic production will have lower risk of bacterial contamination than soil grown product. Analysis of the differences in nutrient composition of both lettuce types revealed differences in Sodium, Zinc and Boron levels. These nutrients were in significantly higher concentrations ($p < 0.05$) in soil grown lettuces. There were no differences in all other nutrients tested. Further, 16S rRNA sequencing revealed differences in the bacterial community associated with both lettuce types. Soil grown lettuce was dominated by *Bacillus* spp whereas hydroponic grown lettuce was dominated by *Pseudomonas* spp. The survival and growth of *L. monocytogenes* may be influenced by the microflora naturally found on the lettuce surface (Söderqvist et al., 2017). For example, *Pseudomonas* are very good biofilm formers and therefore compete with other bacteria by reducing their ability to form biofilms (Norwood & Gilmour, 2001).

The study also revealed a higher microbial diversity in hydroponic grown lettuce than in soil grown lettuce. This is an important point to consider with regards to pathogen colonisation because the competition for nutrients and space on a habitat with high diversity may reduce pathogen colonisation (Klerks et al., 2007). Overall, these differences between the two lettuce types justified further investigations into *L. monocytogenes* attachment, survival and biofilm

formation on both hydroponic and soil grown lettuce with emphasis on whether these differences can influence *L. monocytogenes* growth.

In chapter five, the differences between soil grown and hydroponic grown lettuce with regards to *L. monocytogenes* attachment and growth was explored. Three *L. monocytogenes* strains (O8A06, O8A07 and O8A08) which had been isolated from fresh produce were used. There was no significant difference between the two lettuce types in terms of attachment, survival and growth of *L. monocytogenes*. Also, there were no significant differences between the three different strains that were used either in terms of attachment, survival or growth on lettuce. However, some interesting novel findings were seen. For the first time, we saw *L. monocytogenes* strongly attaching to lettuce surfaces under very minimal exposure times of 1 s and 10 s (Kyere et al., 2019). Washing with sterile distilled water could not completely remove all attached cells. Moreover, there was no significant difference between hydroponic grown and soil grown lettuce leaves for *L. monocytogenes* attachment. It was therefore concluded that any exposure of lettuce leaves to *L. monocytogenes,* even for a short time can result in attachment irrespective of the growth system used. In addition, washing lettuce with water will not eliminate the food safety risk from *Listeria* contamination of lettuce leaves. Since there were no significant differences between soil and hydroponic-grown lettuce leaves with regards to *L. monocytogenes* colonisation, other growth systems such as aeroponics production should also be investigated. In aeroponic production, a sprinkler system sprays the nutrient solution to the roots of the plants. Unlike the hydroponic solution where roots are submerged in the nutrient solution, the roots of aeroponics plants are in constant contact with pure air. Due to this, they have higher oxygen levels leading to proper growth of plants (Klarin et al., 2019).

After attachment, *L. monocytogenes* can survive and grow at 4 and 10°C (temperatures commonly found in lettuce processing). *L. monocytogenes* survival and growth generally decreased in un-bagged lettuce and with increases in lettuce packed in plastic bags. This supports the results of the survey where only bagged lettuces were contaminated with *Listeria*.

In chapter six, lettuce juice was investigated as a potential contributor for *L. monocytogenes* contamination in the processing environment. Many foodborne outbreaks identified the produce processing environment as the source of contamination (McCollum et al., 2013). While the lettuce may provide the primary source of contamination, biofilm growth on processing plant surfaces, enriched by the nutrients from lettuce juice, can provide a secondary source of contamination (Allende et al., 2018). Cutting and washing of lettuce releases juice on processing surfaces (Likotrafiti et al., 2013). As stainless steel is the predominant surface in lettuce processing facilities, an investigation was carried out for the first time the enumerate the biofilm formation of *L. monocytogenes* on stainless steel surfaces exposed to lettuce juice. Lettuce juice supports the biofilm growth of *L. monocytogenes* at 4 and 10°C (Kyere et al., 2020). It was revealed that *L. monocytogenes* forms mixed biofilms with other species found in the lettuce juice. This result calls for the fresh produce industry to consider biofilms that form on processing plant surfaces exposed to lettuce juice when developing pathogen control measures.

In the last part of the study, the ability of UV-C stress to reduce *L. monocytogenes* growth on lettuce was investigated. This was the first time such an approach has been used to reduce *L. monocytogenes* on lettuce leaves. The mechanism behind UV-C stressing of lettuce leaves is an accumulation of polyphenols in plants after stress (Urban et al., 2016). The plant's reaction to the stress is to increase its polyphenol content and some of these compounds are antibacterial with ability to inhibit *L. monocytogenes* attachment and growth (Gamage, 2015). Optimisation

of the UV dose is essential for successful treatment. The results of this study showed an appropriate dose was 1.3 kJm^{-2} while 2.6 kJm^{-2} did not inhibit the attachment and/or growth of *Listeria* but rather damaged lettuce cells. This innovative control method was able to reduce *L. monocytogenes* attachment to lettuce leaves by 1.4-1.5 log CFU/cm^2. Also, it reduced the survival of *L. monocytogenes* by 1.8-1.9 log CFU/cm^2. This reduction is close to the level of contamination found on lettuce in the survey of bagged lettuce in this study. This means that UV stress has the potential to have a real impact in reducing food safety risk associated with *Listeria* contamination of lettuce. Pre-harvest application of UV light in the fresh produce industry needs to be investigated.

The use of UV-C stress together with other washing methods can be useful for reducing microbial load on fresh produce surfaces and therefore should be considered in future fresh produce safety programs. This is supported by researchers who have demonstrated significant reduction of microbial load when UV-C radiation in combination with other methods have been used (Song et al., 2011). The problem with this combined treatment might be due to cost, however, any contamination of leafy greens with pathogens such as *L. monocytogenes* can lead to huge economic loss (Thomas et al., 2015).

The results of this study indicate that other novel non-thermal techniques such as atmospheric cold plasma and LED capable of inactivating microorganisms should also be considered in fresh produce safety. Atmospheric cold plasma are partially ionized gases involving energetic species such as photons, electrons and free radicals. The main advantage of atmospheric cold plasma is that they are cold and therefore its application can be appropriate for fresh produce. In addition, it has been proven to be capable of preserving fresh produce quality (Pankaj et al., 2018). LED has unique properties such as low radiant heat and high monochromatic light emissions. Due to these properties, LED are capable of inactivating microorganisms in fresh

produce. Similar to atmospheric cold plasma, LED are also operated in cold conditions without any thermal damage to the fresh produce (Ghate et al., 2017).

8.2 Highlights or key findings

-This is the first study to compare *Listeria* contamination in bagged and un-bagged lettuces sold in supermarkets. Bagged lettuce poses a higher risk of *Listeria* contamination than un-bagged lettuces, possibly due to the moisture content in bags.

-The study for the first time reported the differences in the bacterial community of soil and hydroponic grown lettuces using 16S rRNA amplicon sequencing. The predominant bacterial genera on hydroponic and soil grown lettuces were *Pseudomonas* and *Bacillus* respectively. Different growth systems in fresh produce cultivation influence the microbiome on the lettuce surface and this has potential to influence pathogen contamination.

-This is the first report to compare the attachment of *L. monocytogenes* on both hydroponic and soil grown lettuce surfaces. *L. monocytogenes* rapidly attaches to both hydroponic and soil grown lettuces under very minimal exposure times.

-This is the first study to report the ability of lettuce leaf extracts or juice to support the biofilm formation of *L. monocytogenes* on stainless steel surfaces and this shows that the fresh produce industry should consider potential biofilms that can be formed on processing plant surfaces exposed to lettuce juice in developing their control programs.

-A new food control method was identified using UV-C light stress to reduce *L. monocytogenes* survival and growth on lettuces. The bacteriostatic property is due to the accumulation of polyphenols in UV-C stressed lettuce.

8.3 Future work

Based on the findings in this thesis, it is recommended that future work include the flowing:

-A more intensive survey of bagged and un-bagged lettuces sold in NZ supermarkets with sampling throughout the entire year to get additional details about *Listeria* contamination in NZ supermarkets.

-Investigate the impact of indigenous bacteria of hydroponic grown lettuce on foodborne pathogens.

-Identify the regulatory mechanisms that trigger the rapid attachment of *L. monocytogenes* to lettuce leaves.

-Examine the effect of *L. monocytogenes* biofilms on mixed species biofilms formed on stainless steel coupons in lettuce juice.

-Investigate, through metabolomics and gene expression, the mechanism of UV-C stress response that reduces *L. monocytogenes* biofilm formation on lettuce.

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