COLONIZATION AND BACTERIOPHAGE BIOCONTROL OF *SALMONELLA* ON FRESH PRODUCE

by

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Abstract

Multiple outbreaks caused by *Salmonella* have been linked to fresh produce. Washing in sanitizing solutions has been shown to reduce microbial populations by <90%. Bacteriophage cocktails have been suggested as an alternative to chemical sanitizers due to their effective and specific antimicrobial activity, safety and lack of effects on organoleptic properties.

Lettuce and tomato plants were separately inoculated with 43 *Salmonella* strains, 26 most commonly associated and 17 not commonly associated with fresh produce outbreaks. *Salmonella* populations were measured immediately after inoculation and after 5 days. Laser Scanning Confocal Microscopy (LSCM) was performed after staining with a fluorescein isothiocyanate (FITC) labelled anti-*Salmonell*a antibody. Romaine lettuce was inoculated with a phage cocktail + 1.0mM calcium chloride or only 1.0mM calcium chloride one day before inoculation with 3 separate *Salmonella* strains. *Salmonella* populations were measured immediately after inoculation and after 1 and 2 days.

Populations of 26 strains (60.5%) increased on all plant species and cultivars, although there were significant differences ($p<0.05$) in the extent of population increase by different strains on the same plant species/cultivar. The remaining strains displayed differential ability to colonize lettuce and tomato plants depending on plant species or cultivar. Most strains not commonly associated with fresh produce outbreaks were able to colonize the plants. LSCM showed that cells or cellular aggregates were located within stomates, in surface depressions adjacent to stomata or in random microsites not associated with specific anatomical features.

Application of a bacteriophage cocktail to Romaine lettuce leaf sections 24 hours before inoculation significantly reduced ($P<0.05$) populations of *Salmonella* Saintpaul S204, Saintpaul S205 and Typhimurium S441 by 2-4 log CFU/cm^2.

The results of this study showed that the interaction between plant host and colonizing *Salmonella* is complex and subject to several interacting factors. Moreover, the colonization potential of *Salmonella* is highly variable and should be carefully considered in the selection of experimental strains for future research on the ecology of this bacterial species on growing food plants.

Lay Summary

Fresh produce has caused multiple *Salmonella* outbreaks in recent decades. Washing in sanitizing solutions has been the most common method employed to reduce human pathogen contamination. However, prior research has shown that sanitizing solutions reduces microbial populations by <90%. As a result, surviving *Salmonella* populations may still be high enough to cause outbreaks and illnesses. To address this food safety concern, the fate of 43 *Salmonella* strains were examined on lettuce and tomato pre-harvest plants to determine if particular strains are of increased concern on plant leaves, and the use of bacteriophages (viruses targeting bacteria) as an alternative measure to sanitizing solutions was investigated. This study provided new insight into the behavior of *Salmonella* on pre-harvest plants and the effectiveness of bacteriophages, which is important in the development of better strategies to reduce foodborne outbreaks related to fresh produce.

Preface

Subsections 2.2.1 Lettuce and tomato plant propagation, 2.2.3 Whole plant inoculation and measurement of *Salmonella* populations, 3.2.3 Bacteriophage application to inoculated Romaine lettuce were based on protocols designed by Dr. Pascal Delaquis at the Summerland Research and Development Center of Agriculture and Agri-Food Canada. The author, Catherine Wong, was responsible for carrying out the laboratory work and performing adjustments to inoculation levels, plant sample sizes and biological replicates to fulfil research objectives.

The rest of this research was completed solely by the author, Catherine Wong, under the guidance of Dr. Siyun Wang.

The work in this thesis is original. Research conducted in Chapter 2, subsections 2.2.1 – 2.2.5 have been submitted, accepted for publication and is currently in press to be published as Wong C, Wang S, Levesque RC, Goodridge L, Delaquis P (2019) Fate of 43 *Salmonella* sp. Strains on Lettuce and Tomato Seedlings in Journal of Food Protection. The rest of the work in this thesis have not been previously published.

Table of Contents

List of Tables

List of Figures

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Chapter 1. Introduction and Literature Review

1.1 The role of *Salmonella* in foodborne illness and produce-associated outbreaks

Non-typhoidal *Salmonella* (NTS) are an on-going burden on society due to the number of illnesses, hospitalizations and deaths caused by the bacterium. In Canada, yearly estimates place NTS as fourth for the highest number of illnesses, second for number of hospitalizations and third for number of deaths caused by bacteria, parasites or viruses (Thomas et al. 2015). In the US, NTS placed second for highest number of illnesses and first for number of hospitalizations and death annually (Scallan et al. 2011).

Salmonellae are Gram-negative, rod-shaped, facultative anaerobic bacteria that are motile by means of a peritrichous flagellum (Andino and Hanning 2015). The genus *Salmonella* contains two species: *Salmonella enterica* (*S. enterica)* and *S. bongori* (Grimont and Weill 2007). *S. bongori* is predominately associated with cold-blooded animals (Fookes et al. 2011). *S. enterica* is further categorized into 6 subspecies: *enterica, salamae, arizonae, diarizonae, houtenae* and *indica* (Grimont and Weill 2007). The species *S. enterica* is comprised of more than 2,600 serovars, all of which are capable of causing disease in humans (Fookes et al. 2011). One group of serovars including *S.* Typhi, Paratyphi A, Paratyphi B and Paratyphi C are causative agents of life-threatening enteric fever (also known as typhoid or paratyphoid fevers) (Parry et al. 2002; Näsström et al. 2014). The term NTS refers collectively to *Salmonella* that cause salmonellosis, a mild to severe gastrointestinal illness (Phuong et al. 2017). National *Salmonella* surveillance data is compiled from laboratory-confirmed *Salmonella* strains involved in causing foodborne illnesses in humans (Centers for Disease Control and Prevention 2011). Laboratories routinely serotype *Salmonella* strains recovered from clinical cases (Centers for Disease Control and Prevention

2011). Examples of *Salmonella* serovars include *S.* Enteriditis and *S.* Typhimurium, both of which are common causes of salmonellosis throughout the world (Tauxe 1999).

Fresh produce is recognized as a leading vehicle for the foodborne transmission of *Salmonella* in the US, Canada and the European Union (Greig and Ravel 2009; Ravel et al. 2009; Callejón et al. 2015). Four *Salmonella* outbreaks linked to fresh produce were reported in the US in 2018 (Centers for Disease Control and Prevention 2018) Recent fresh produce outbreaks in the US have been caused by a variety of fresh produce commodities, including pre-cut melon and raw sprouts (Centers for Disease Control and Prevention 2018). In the 10-year period from 1998 – 2008 tomatoes and leafy vegetables were the cause of 5% and 2.5% of all outbreaks associated with the consumption of fresh produce in the US (Jackson et al. 2013). *Salmonella* serovars linked to tomato outbreaks that occurred over this time period included *S.* Newport (32%), *S.* Typhimurium (16%), *S.* Braenderup (11%), *S.* Enteritidis (11%) and *S.* Javiana (11%) (Jackson et al. 2013). *S.* Montevideo and *S.* Baildon were also implicated in significant outbreaks (Bennett et al. 2015). Moreover, numerous outbreaks reported between 1998 – 2008 were associated with leafy-green vegetables and half were ascribed to *S.* Newport (30%) and Javiana (20%) (Jackson et al. 2013). Overall, 13 of the more than 2,500 known *Salmonella* serovars have caused the majority of salmonellosis associated with fresh produce, including Javiana, Newport, Poona, Muenchen, Mbandaka, Senftenberg, Litchfield, Thompson, Montevideo, Saintpaul, Agona, Typhimurium and Enteritidis (Jackson et al. 2013; Andino and Hanning 2015). Cooking is a common and effective method for the elimination of bacterial enteric pathogens in foods. It has been suggested that increasing consumption without prior cooking is contributing to the increase in foodborne illnesses associated with fresh produce (He et al. 2011).

1.2 Sources of *Salmonella* in the agricultural environment

Several potential sources of *Salmonella* in the agricultural environment have been described. Gu et al. (2018) examined irrigation water drawn from an irrigation pond and a well in a tomato growing area of the US. *Salmonella* was present in 64.7% of water samples from the irrigation pond between August and December (Gu et al. 2018). The average *Salmonella* density was found to be 4.06 ± 1.86 most probable number (MPN)/L and 2.30 ± 1.10 MPN/L in two consecutive years (Gu et al. 2018). The results highlighted temporal differences in the prevalence of *Salmonella* in pond water but there was no significant correlation with temperature or rainfall (Gu et al. 2018). Moreover, *Salmonella* was not detected in irrigation well water (Gu et al. 2018). *Salmonella* was isolated from tomato plants irrigated with pond water during every growing season over the study's 4-year time frame (Gu et al. 2018). The same study also showed that production practices (staking with mulch, staking and free growth without mulch) also affected the likelihood of *Salmonella* detection on tomato plants, although the level varied monthly from year to year (Gu et al. 2018). Collectively, these results highlighted the role of irrigation water and agricultural practices on the risk of crop contamination.

Soil can potentially serve as a source of *Salmonella* that could migrate to growing plants. Zheng et al. (2013) showed that tomato plants grown in soil contaminated with a *Salmonella* cocktail at ~1 x 10^8 CFU/g became contaminated with multiple *Salmonella* serovars that migrated from the soil line up to 10 cm inside the tomato plant stem. The recovery of *Salmonella* in stem segments was strain-dependent and strains of *S.* Newport and *S.* Montevideo were found in different areas of the stem (Zheng et al. 2013). Hintz et al. (2010) similarly found that tomato plants irrigated with 7 log CFU/ml *S.* Newport were tested positive for the pathogen in the roots

and stems. However, the *S.* Newport strain was not consistently found in the leaves or fruits of the tomato plants (Hintz et al. 2010). In contrast, one group of researchers was unable to detect *Salmonella* in the stems or in the fruits of tomato plants grown in soil containing 2.3 – 3.7 log CFU/g applied in irrigated water (Jablasone et al. 2004). These results could suggest that high levels (7 or 8 log CFU/g) in soil may be necessary for *Salmonella* migration to the plant. It also increasingly clear that pathogens such as *Salmonella enterica* (*S. enterica*) can survive in soil for long periods of time and retain the ability to colonize plants. Barak and Liang (2008) showed that *S. enterica* populations decreased ~1 log CFU/g in soil after 3 weeks but stabilized between 3-5 weeks when the populations were reduced by ~1 CFU/g. This research also showed that contaminated plant debris left in the field for continuous plant cropping can re-contaminate new crops (Barak and Liang 2008). *S. enterica* was recovered from both the phyllosphere (above soil line) at ~5 log CFU/g and the rhizoplane (below soil line) at ~3 log CFU/g from seeds sown 24 hours after contaminated plant debris incorporation in the soil (Barak and Liang 2008). In contrast, *S. enterica* was not detected in the phyllosphere and populations < 100 CFU/g were measured in the rhizoplane of plants grown from seeds sown 7 days after plant debris incorporation (Barak and Liang 2008). These findings illustrated the role of field management practices on the risk of crop contamination with *Salmonella.*

Other factors that can contribute to *Salmonella* contamination during pre-harvest include animals, insects and human handling (Olaimat and Holley 2012). Flies, especially species *Musca domestica* are prevalent on farms and can be carriers of *Salmonella*, resulting in contamination of plant crops (Zamora-Sanabria and Alvarado 2017). The contamination cycle continues as animals, both wild and domesticated, can become infected after consumption of contaminated flies

(Zamora-Sanabria and Alvarado 2017). Aside from flies, *Dermanyssus gallinae*, *Ornithonyssus sylviarum* and *Ornithonyssus bursa* are species of mites generally found in animal manure and feed that can transmit *Salmonella* to animals or food crops (Zamora-Sanabria and Alvarado 2017). Wild animals such as birds and small mammals have also been shown to spread *Salmonella* from farm to farm (Zamora-Sanabria and Alvarado 2017). Unhygienic workers could introduce pathogens into the food system through reduced instances of hand washing as pathogens are present in the nose, hair, throat, clothes, shoes and intestines of humans (Andres and Davies 2015; Zamora-Sanabria and Alvarado 2017). Poorly cleaned and sanitized equipment and transport containers are also recognized as potential sources of human pathogens that can cause repeated instances of contamination in short periods of time (Beuchat 2002).

1.3 Ecology of *Salmonella* on food plants

Considerable research directed at understanding the ecology of *Salmonella* on food plants has indicated that several interacting extrinsic environmental factors may influence the fate of the species. A wide range of *Salmonella* strains and experimental conditions have been employed in research intended to characterize the behavior of the species in fresh produce commodities at various stages along production-to-consumption chains. For example, *Salmonella* has been shown to grow on alfalfa, fenugreek, lettuce and tomato sprouts and seedlings propagated from contaminated seed (Cui et al. 2018). Growth has also been demonstrated on the leaves of lettuce plants propagated in growth cabinets maintained at 28°C, however the rate and extent of population increase were higher on young leaves containing higher free nitrogen content than middle, older leaves (Brandl and Amundson 2008; Brandl et al. 2013). *Salmonella* Newport, a serovar associated with a 2005 multistate tomato outbreak in the United States, has been shown to grow and survive

on tomato fruits better than *Salmonella* Typhimurium, but the latter was able to colonize seedling leaves better than *Salmonella* Newport (Greene et al. 2008; Han and Micallef 2014). Collectively, this research indicates that the fitness and ultimate fate of *Salmonella* on both aerial or subterranean organs of growing edible plants is affected by a range of intrinsic environmental factors including commodity or cultivar specific characteristics, ambient temperature, water activity, nutrient availability and plant-derived metabolites (Brandl et al. 2013; Wiedemann et al. 2014; Han and Micallef 2016). Comparatively less is known about the influence of intrinsic serotype or strain-associated traits on the ecology of *Salmonella* on food plants.

Anatomical features on the surface of plants are primarily meant to lessen water losses and prevent dehydration (Kerstiens 1996; Riederer and Schreiber 2001). The cuticle, a lipophilic layer that coats all aerial organs, minimizes loss of water that is not lost through the stomata (Kerstiens 1996; Riederer and Schreiber 2001). Epicuticular waxes that coat the outer surface of the cuticle are water-repellent and help to keep the plant surface dry and clean (Jeffree 2006; Burton and Bhushan 2006). Some anatomical features, such as stomates or sites where sub-cutaneous tissues are exposed due to damage caused by insects or mechanical injury can provide attachment sites (Seo and Frank 1999; Burnett et al. 2000; Takeuchi and Frank 2000; Liao and Cooke 2001; Kroupitski et al. 2009a). For example, it has been shown that viable cells of *Salmonella* Typhimurium (*S.* Typhimurium) can attach to various surface structures but primarily to the cell walls of potato (Saggers et al. 2008). The results of this study suggested that pectin in the cell wall may favor attachment as a reduction in pectin led to a reduction in *S.* Typhimurium attachment (Saggers et al. 2008).

The behavior of *Salmonella* after attachment has been reported to vary depending on the site being colonized. Zheng et al. (2013) found that *Salmonella* concentrations increased on tomato blossoms from 1.12 x 10^5 to 1.77 x 10^5 CFU/blossom after 7 days post inoculation. In contrast, populations did not change on tomato leaflets for 8 days post inoculation and decreased thereafter (Zheng et al. 2013). Work by these researchers also showed that 70% of tomato fruit that developed from inoculated blossoms were positive for *Salmonella* and that contamination could spread to adjacent, non-inoculated fruit (Zheng et al. 2013).

Brandl and Amundson (2008) have found *Salmonella* growth to be dependent on the age of lettuce leaves after the aerial part of whole lettuce plants were inversely inoculated. The growth of *S. enterica* was seven fold higher on young leaves (inner rosette of lettuce plants with leaves <4cm long) compared to middle leaves (5th and 6th leaves of the lettuce plant) 48 hours after inoculation (Brandl and Amundson 2008). On old leaves (1st and 2nd leaves of the lettuce plant), *S. enterica* populations varied between replicates but were generally lower than on the young leaves (Brandl and Amundson 2008). Similar *S. enterica* populations observed in lettuce plants were also observed in mature lettuce (Brandl and Amundson 2008). It is possible that the higher growth of *S. enterica* on the young leaves is related to the higher populations of native bacteria present compared to middle and old leaves (Brandl and Amundson 2008). Kroupitski et al. (2009b) found *S.* Typhimurium to prefer attachment to cut leaf edges or injured leaf tissues over intact tissues. This finding suggests that food producers and consumers should handle lettuce and other fresh produce with care to prevent mechanical injury that could promote *Salmonella* attachment and growth to potentially harmful levels.

1.4 Control of *Salmonella* on food plants

Given the potential for contamination with human pathogens during the production of food plants, means to reduce their populations are highly desirable. Current approaches to pathogen removal or inactivation in the fresh produce industry have relied on washing in sanitizing solutions (Beuchat 1998; Brackett 1999; Sapers 2001). However, these methods have been shown to reduce microbial populations by < 90%, potentially leaving enough bacterial cells to grow and cause foodborne outbreaks (Beuchat 1998; Brackett 1999; Sapers 2001). Sanitizers that have been approved for use in the food industry include chlorine/hypochlorite, chlorine dioxide, acidified sodium chloride, iodine and quaternary ammonium compounds (U.S. Food & Drug Administration 2014). Chlorine is one of the widely used sanitizers and has been used in the food industry for several decades (U.S. Food & Drug Administration 2014).

The efficacy of different sanitizers on bacterial inactivation is variable. Banach et al. (2017) examined the antimicrobial effects of sodium hypochlorite, aqueous chlorine dioxide and a silver-copper antimicrobial solution against *S.* Typhimurium in wash water and on lettuce tissues. At 37°C, both 10 mg/L of sodium hypochlorite and 5 mg/L aqueous chlorine solutions decreased *S.* Typhimurium populations in water by 4 log CFU/mL within seconds (Banach et al. 2017). The silver-copper antimicrobial solution with 9.1-9.9 mg/L silver and 1.2 mg/L copper required 10 minutes before populations were reduced by 4 log CFU/mL (Banach et al. 2017). Washing of lettuce tissues in portable water alone had little effect on *S.* Typhimurium, and the sodium hypochlorite, aqueous chlorine dioxide solution and silver-copper antimicrobial solution reduced populations by < 2 log CFU/g at both 5°C and 20°C (Banach et al. 2017). Currently, a reduction

of 2 – 5 log CFU in bacterial population is acceptable for post-harvest washing (Gombas et al. 2017; Murray et al. 2017).

In another study, chopped and un-chopped parsley inoculated with *S.* Typhimurium was washed with water, 4% acetic acid vinegar, 0.25 g/L sodium dichloroisocyanurate and a 1,000 ppm chlorine solution at 5°C for 24 hours (Faour-Klingbeil et al. 2016). Chlorine solutions induced the highest population reduction (2.27 log CFU/g), followed by sodium dichloroisocyanurate (1.85 log CFU/g), vinegar (1.25 log CFU/g) and water (0.98 log CFU/g). Tan et al. (2015) washed turnips in solutions with 80-100 ppm acidic electrolyzed water, 1200 ppm acidified sodium chlorite, 1% cetylpyridinium chloride, 200 ppm chlorine, 2 ppm ozonated water and 150 ppm sodium dichloroisocyanurate. Acidified sodium chlorite led to the highest reduction in *Salmonella* populations (~3.5 log CFU/turnip), followed by acidic electrolyzed water (2.5 log CFU/turnip), sodium dichloroisocyanurate (~2.2 log CFU/turnip) and cetylpyridinium chloride and chlorine with ~1.5 log CFU/turnip each (Tan et al. 2015). Ozone and water were marginally effective, and populations were reduced by only ~0.3 log CFU/turnip (Tan et al. 2015). In the examples above, wash water sanitizers generally reduced but did not completely eliminate *Salmonella.* At the present time, none of the sanitizers available for commercial use can eliminate pathogens on fresh produce to prevent outbreaks of foodborne illnesses.

Biofilm formation, attachment site inaccessibility, strength of attachment and internalization may also contribute to the limited efficacy of sanitizers applied in wash water (Costerton 1995; Carmichael et al. 1998; Seo and Frank 1999; Burnett et al. 2000; Fett 2000; Takeuchi and Frank 2000; Takeuchi et al. 2000; Liao and Cooke 2001; Sapers 2001). Biofilm

formation leads to cellular aggregation and the protection of bacterial cells below the outermost layer due to lack of access by the sanitizing solution (Costerton 1995; Carmichael et al. 1998; Fett 2000; Sapers 2001). Food plant surfaces are not generally even and smooth throughout, and bacterial cells tend to localize in cracks and crevices (Seo and Frank 1999; Burnett et al. 2000; Takeuchi and Frank 2000; Liao and Cooke 2001; Sapers 2001). Such areas may similarly be inaccessible to sanitizers (Sapers et al. 2000; Sapers 2001). The possibility of internalization of enteric pathogens during the growth of the plant would also affect the performance of sanitation treatments (Sapers 2001; Kroupitski et al. 2009a). Erickson et al. (2018) examined internalization of *Salmonella* in 8 lettuce cultivars (Gabriella, Green Star, Romaine, New Red Fire, Starfighter, Tropicana, Two Star and Muir) grown under greenhouse conditions and found that internalization could occur, although the extent was highly cultivar dependent. Evidently, internalized bacteria would remain fully sheltered from contact with sanitizing solutions during washing.

Contamination with *Salmonella* (or other enteric bacterial pathogens) can occur at any stage during the farm-to-fork chain. Current intervention strategies are primarily intended to reduce, rather than eliminate, such pathogens at the end of the chain, normally just prior to retail distribution (Erickson et al. 2018). Depending on the level of contamination at the outset, such strategies may not provide sufficient reductions of pathogens to prevent foodborne infections. Therefore, the control of contamination at early (i.e., preharvest) stages could clearly lessen overall risk. There are currently no known chemical or physical treatments that can achieve this goal in growing food plants. Consequently, alternative approaches based on biocontrol principles using antagonistic bacteria or bacteriophages should be considered.

1.5 Bacteriophages

Bacteriophages are viruses that infect bacteria but are generally harmless to humans, animals and plants (Sillankorva et al. 2012). Bacteriophages can infect bacterial cells by a chance encounter in the environment (Koskella and Meaden 2013). The infected bacterium becomes the host in which the bacteriophage reproduces, leading to the lysis of the bacterium and release of viral progeny termed virion in a lytic reproduction cycle (Koskella and Meaden 2013; Howard-Varona et al. 2017; Rodríguez-Rubio et al. 2017). While most bacteriophages tend to infect closely related strains within a bacterial species, i.e. have a fairly narrow host range, there are examples of broad host range bacteriophage capable of infecting several species within a genus or even several genera (Flores et al. 2011).

1.5.1 Bacteriophage reproduction

The lytic cycle is the most common means of bacteriophage reproduction (Koskella and Meaden 2013). The newly released virions can infect other bacterial cells and start lytic cycles of their own (Howard-Varona et al. 2017). Some bacteriophages known as temperate phages can undergo either a lytic or a lysogenic cycle (Howard-Varona et al. 2017). The lysogenic cycle is comprised of three steps (Howard-Varona et al. 2017). The first step is termed establishment, wherein the temperate phage determines whether it will enter the lytic cycle and produce virions or if it will enter the lysogenic cycle as a prophage by infecting a bacterial cell and incorporating the phage DNA into the genome of the host (Howard-Varona et al. 2017). The mechanisms underlying the decision to enter the lysogenic cycle are not fully understood (Casjens and Hendrix 2015). Research with the *E.coli* phage λ has shown that three factors influence the path to lysogeny: (i) genetic compatibility, (ii) host physiological state, and (iii) phage density (Casjens and Hendrix

2015). Genetic compatibility refers to the presence of integration sites in the host, such as the *attB* integration site (Casjens and Hendrix 2015). Host physiological state due to stresses such as nutrient depletion can lead to increased chances of lysogeny (Casjens and Hendrix 2015). The ratio of bacteriophages to bacterial host, which is usually defined as the multiplicity of infection (MOI), can similarly influence the onset of lysogeny (Abedon 2016).

The second step in lysogeny, maintenance, refers to the series of events leading to the replication of the bacterial host genome with integrated bacteriophage DNA (Howard-Varona et al. 2017). This precedes the third and final induction step wherein the infected bacterial host cell divides into two daughter cells each carrying the bacteriophage DNA (Howard-Varona et al., 2017). At this stage, a switch from the lysogenic cycle to the lytic cycle can occur if there is a low ratio of bacterial cells to bacteriophages (Czyz et al. 2001) or if there are external stressors that trigger the SOS response or the DNA damage response of the bacterial cell (Casjens and Hendrix 2015). Examples of external stressors include pH, temperature or nutrient changes, antibiotics, unfamiliar DNA, hydrogen peroxide or agents causing damage to DNA (Cochran et al. 1998; Banks et al. 2003; Mell and Redfield 2014; Casjens and Hendrix 2015; Howard-Varona et al. 2017). The presence of nearby bacteriophage may also affect the lysogenic to lytic switch (Howard-Varona et al. 2017). Once switching is initiated, the bacteriophage DNA is excised from the bacterial host chromosome and replicates, creating virion particles (Howard-Varona et al., 2017). The last step of the lysogenic to lytic switch can occur spontaneously rather than by induction, and only some of the lysogenic bacteriophages enter the lytic cycle (Howard-Varona et al., 2017). When this occurs, a mixed population of both lysogenic and lytic bacteriophages is created (Howard-Varona et al., 2017).

1.5.2 Bacteriophages and their potential use as biocontrol agents in food and on food plants

The anticipated efficacy of bacteriophages has led to considerable research on potential applications for the control of enteric bacterial pathogens at both the pre-harvest and post-harvest stages (Moye et al. 2018). Some research has been directed at potential applications for the control of *Salmonella* on fresh produce. Leverentz et al. (2001) applied an experimental cocktail consisting of four bacteriophages to fresh-cut fruit. The cocktail reduced *Salmonella* populations on cut honeydew melon by ~2.5 log CFU/g at 20°C and ~3.5 log CFU/g at 5°C and 10°C (Leverentz et al. 2001). However, the cocktail had no effect against *Salmonella* on apple slices (Leverentz et al. 2001). Other studies have shown that bacteriophage cocktails can reduce *Salmonella* populations on sprouted vegetables (~3 log CFU/g, Ye et al. 2010) and on fresh-cut lettuce (2-4 log CFU/cm^2, Spricigo et al. 2013). Several bacteriophage preparations have been approved for use in foods and are now commercially available. For example, SalmoPro™ gained regulatory approval from the US Food and Drug Administration (USFDA) with a designation of Generally Recognized as Safe (GRAS) (US Food and Drug Administration 2015). SalmoPro™ has been approved for use in post-harvest fruits and vegetables and in poultry, red meat, eggs, fish and shellfish (US Food and Drug Administration 2015). All commercial products consist of lytic bacteriophages isolated from natural sources as the use of genetically modified bacteriophages has not gained regulatory approval (Moye et al. 2018).

A common observation derived from studies on bacteriophage usage in foods is that the decrease in targeted bacterial populations is not consistent (Guenther et al. 2012; Kang et al. 2013; Spricigo et al. 2013; Moye et al. 2018). Most of the inactivation of targeted bacterial populations happens upon first contact with the bacteriophages with little or no further reduction afterwards

(Guenther et al. 2012; Kang et al. 2013; Spricigo et al. 2013; Moye et al. 2018). Moreover, reductions of targeted bacteria in solid foods generally range between 1-3 log CFU/g and complete elimination is rare (Leverentz et al. 2001; Whichard et al. 2003; Higgins et al. 2005; Ye et al. 2010; Moye et al. 2018). This is somewhat unexpected as each phage replication cycle should result in a high number of released virions to infect surviving bacteria. A few studies have suggested that repetitive lytic bacteriophage replication cycles does not occur under current conditions used in some experimental systems (Soni et al. 2012; Chibeu et al. 2013; Oliveira et al. 2014; Moye et al. 2018). Limited moisture and the lack of bacteriophage motility may have been responsible in some cases (Hudson et al. 2010; Moye et al. 2018). In moist food matrices such as beverages or sliced watermelons, water can serve as a medium for the transport of bacteriophages across food surfaces to facilitate access to target bacteria (Hudson et al. 2010; Moye et al. 2018). Conversely, limited transport in dry food matrices results in restricted access to bacterial targets (Hudson et al. 2010; Moye et al. 2018). Potential options to increase bacteriophage efficacy in dry food matrixes include the use of higher concentrations of phage particles and application in solutions (Bower and Daeschel 1999; Atterbury et al. 2003; Leverentz et al. 2004; Abuladze et al. 2008; Moye et al. 2018). However, higher concentrations could result in higher costs to food processors and application in solutions may necessitate drying after treatment (Moye et al. 2018).

Since available bacteriophage preparations can only reduce but not eliminate target bacterial contaminants, their use can be considered in conjunction with other antimicrobial strategies. Given that most enteric bacterial pathogens do not grow at temperatures <4°C, efficacy may be enhanced in foods that require refrigeration or freezing (Guenther et al. 2012; Endersen et al. 2013; McLean et al. 2013; Moye et al. 2018). Other preservation methods

employing chemical sanitizers or irradiation could also enhance overall efficacy, although both can inactivate bacteriophages and have to be applied in a timely sequence to achieve the desired reduction (Moye et al. 2018). For example, Magnone et al. (2013) showed that an additional reduction of up to 2 log CFU/g could be achieved on broccoli, cantaloupe and strawberries when bacteriophage treatment was followed by a levulinic acid wash.

In parallel with chemical sanitizers, bacteria can gain resistance to bacteriophages thereby rendering treatments less ineffective over time (Hong et al. 2016; Moye et al. 2018). The formulation of cocktails consisting of several bacteriophages, preferably from different taxonomic families, is currently the main approach used to combat resistance (Moye et al. 2018). The processing stage at which bacteriophage treatment is added is also believed to influence the potential for bacteriophage resistance (Moye et al. 2018). It has been suggested that application immediately before packaging rather than earlier stages in a food process reduces the risk of resistance because fewer bacterial targets are present (Moye et al. 2018).

Despite considerable interest, bacteriophages are currently not widely used for the control of human pathogens and the enhancement of food safety. Optimal concentration testing of bacteriophage particles to achieve necessary coverage in a food product can be a challenge. Commercial bacteriophage preparations require refrigerated storage (2-8°C) as concentrations decrease over time. (Moye et al. 2018). This can introduce some uncertainty about the concentration of infectious particles at specific points in time. Testing for optimal concentrations also requires facilities and equipment, skilled technical resources and time required to perform the testing, which introduces costs that may be prohibitive for smaller processors. In addition, the

latter are generally reluctant to modify their food processing procedures to accommodate a new treatment that may potentially cost more than their current practices (Moye et al. 2018). There is also the added concern that consumers may not readily accept the notion of food producers adding "viruses to their food" (Moye et al. 2018). However, the use of bacteriophage as preservatives in food is well aligned with growing consumer interest in "natural foods" that have not been genetically modified, treated with antibiotics or chemical sanitizers, are organic and produced locally (Reganold and Wachter 2016; Moye et al. 2018).

Bacteriophage usage is not exclusive against pathogens. Li et al. (2014) found concentrations of 10^6 plaque forming units (pfu)/mL and 10^8 pfu/mL of bacteriophage Spp001 to be an effective biocontrol method against *Shewanella putrefaciens*, a spoilage bacterium in chilled flounder fillets. Spp001's effectiveness against *S. putrefaciens* led to longer extensions in shelf life of chilled flounder fillets compared to 5g/L of potassium sorbate, a chemical preservative (Li et al. 2014). Depending on the bacteriophage chosen and its target, bacteriophages can act against pathogens and spoilage-organisms. Bacteriophages are an excellent choice for preservatives because they do not alter the sensory characteristics of food (Sillankorva et al. 2012).

1.5.3 Bacteriophage stability

The stability of bacteriophages may depend on the storage buffer solution. Adams (1949) found bacteriophage T5 to be most stable in calcium ion solution. Bacteriophage T5 lost its activity when stored in phosphate buffer and was inactivated when stored in citrate solution (Adams 1949). MS2 bacteriophage was tested in 4 different salt solutions in the range of 10 mM - 1.0 M: lithium chloride (LiCl), sodium chloride (NaCl), potassium chloride (KCl) and calcium

chloride ($CaCl_2$) (Mylon et al. 2010). Increasing concentrations of calcium chloride led to increases in bacteriophage aggregation caused by the neutralization of the negatively charged moieties on the surface of the bacteriophage (Mylon et al. 2010).

1.6 Research purpose, hypothesis and objectives

The potential for lesser known *Salmonella* serovars or strains to attach, persist or grow on food plants and whether serovar or strain-associated differences affect these behaviours are currently unclear. A better understanding of these factors is needed to guide new strategies for the control of *Salmonella* on growing food plants, notably strategies based on the use of bacteriophages. Consequently, the first hypothesis was that the fate of *Salmonella* on tomato and lettuce pre-harvest plants is plant, serovar and/or strain specific. The first objective of this study was to determine the fate of *Salmonella* strains on growing lettuce and tomato plants. A total of 43 *Salmonella* strains comprised of 26 strains most commonly associated and 17 not commonly associated with fresh produce outbreaks were selected. Each strain was separately inoculated onto 2 lettuce and 2 tomato cultivars and *Salmonella* populations were measured immediately after inoculation (Day 0) and 5 days after inoculation (Day 5).

In a second hypothesis, the attachment and colonization sites of *Salmonella* on tomato and lettuce plants are comprised of stomates and/or areas near stomates. The second objective was to use Laser Scanning Confocal Microscopy (LSCM) to examine the surface of lettuce and tomato plants to identify sites colonized by *Salmonella*.

The third hypothesis was that the use of *Salmonella* bacteriophages will decrease *Salmonella* populations on a plant surface. The third objective was to determine the efficacy of bacteriophages against *Salmonella* on a plant surface. To this end, a bacteriophage cocktail comprised of 5 broad host range bacteriophages was applied to lettuce leaves before 3 individual *Salmonella* strains were inoculated onto the leaves.

Chapter 2. Fate of *Salmonella* on tomato and lettuce plants and the identification of sites colonized by *Salmonella* using microscopy

2.1 Introduction

Salmonella is one of the top pathogens that cause outbreaks of foodborne illness (Centers for Disease Control and Prevention 2011; Scallan et al. 2011). In 2018 alone, multiple *Salmonella* strains were responsible for 16 known outbreaks in the US (Centers for Disease Control and Prevention 2019). Two outbreaks associated with dried coconut and chicken salad were caused by *S.* Typhimurium (Centers for Disease Control and Prevention 2019). *S.* Newport was responsible for two other outbreaks due to contamination of ground beef and frozen shredded coconut (Centers for Disease Control and Prevention 2019). Four of the 16 outbreaks (25%) were associated with plant-based foods, including frozen shredded coconut, raw sprouts, dried coconut and pre-cut melon (Centers for Disease Control and Prevention 2019). This brings into focus a food commodity that could potentially require extra measures to reduce the risk of foodborne outbreaks and illnesses.

Fresh produce is one of the main food commodities that is widely consumed raw or without a heat treatment or kill step to inactivate human pathogens. Consequently, the safety of these foods relies on thoroughness of cleaning and sanitation and the use of non-thermal treatments. However, cleaning and sanitation are not always effective and *Salmonella* populations in a single lot or batch may remain high enough to cause illnesses or outbreaks. Non-thermal treatments, such as washing in chlorinated water cannot eliminate *Salmonella* due to

biofilm formation, attachment site inaccessibility, strength of attachment or internalization of the pathogen (Costerton 1995; Carmichael et al. 1998; Seo and Frank 1999; Burnett et al. 2000; Fett 2000; Takeuchi and Frank 2000; Takeuchi et al. 2000; Liao and Cooke 2001; Sapers 2001).

In the past 15 years, there have been at least 2 major *Salmonella* outbreaks caused by tomatoes (Greene et al. 2008; Barton Behravesh et al. 2012; Centers for Disease Control and Prevention 2018). In 2006, a *S.* Typhimurium multistate tomato outbreak caused 189 cases in 21 states in USA and 1 case in Canada from a patient who traveled to the US (Barton Behravesh et al. 2012). The outbreak was traced to a tomato packing house in Ohio that may have been contaminated by irrigation water in nearby creeks, wells and ditches (Barton Behravesh et al. 2012). Tomatoes contaminated by *S.* Newport caused two outbreaks responsible for 510 cases in 2002 and 72 cases in 2005 (Greene et al. 2008). In both cases, the tomatoes were traced to a farm where crops were grown with irrigation water contaminated with the same strain (Greene et al. 2008).

In the 10-year time period from 1998-2008, leafy vegetables were the cause of 2.5% of all *Salmonella* outbreaks associated with the consumption of fresh produce in the US (Jackson et al. 2013). The percentage is alarming because the commodity is almost always consumed without prior heat treatment. Other food commodities responsible for a high number of outbreaks include eggs and chicken (Jackson et al. 2013). Eggs and chickens are generally consumed after heat treatments and an increase in public education on thorough cooking has undoubtedly helped to limit the number of *Salmonella* infections caused by these food commodities; unfortunately, this is not an option for leafy vegetables.

Previous research suggests that bacterial human pathogens like *Salmonella* preferentially colonize specific sites on the leaves of food plants. Stomates and areas immediately adjacent to stomates have been shown to be actively colonized and one report suggests that *Salmonella enterica* serovar Typhimurium can exploit open stomates for entry into the inner tissues of iceberg lettuce (Kroupitski et al. 2009b). Barak et al. (2011) identified trichomes as a preferred colonization site for *S.* enterica on tomato leaves. Brandl and Amundson (2008) reported a similar finding for another bacterial pathogen, *Escherichia coli* 0157:H7, on lettuce leaves. In addition, cells were also found on the veins and between veins on the surfaces of younger lettuce leaves and large aggregates of cells were mainly found on the surfaces of middle leaves.

Most studies concerning the ecology of *Salmonella* on food plants and foods derived from them have been conducted with a limited number of strains from a narrow range of serovars, primarily isolates recovered during investigations of produce-associated outbreaks. Consequently, comparatively little is known about intrinsic serovar or strain-associated differences in the plant colonization potential of *Salmonella*. The present experiments were conducted to examine the fate of 43 strains drawn from a range of serovars subsequent to inoculation on plants from two lettuce and tomato cultivars. This work was also conducted with a view to inform the selection of *Salmonella* strains that successfully colonize food plants to serve as "targets" in experiments intended to assess the efficacy of bacteriophage for their control in the second part of the study. Studies aimed at the identification of sites colonized by *Salmonella* have been performed with a limited number of strains and have typically been done on mature plants. The objective described in this chapter was to identify sites colonized by several strains of *Salmonella* on developing lettuce and tomato seedlings.

2.2 Materials and Methods

2.2.1 Lettuce and tomato plant propagation

Tomato seed from cultivar Amish Paste and lettuce seed cultivars Parris Island Cos and Winter Density were obtained from West Coast Seeds (BC, Canada). Tomato seed from cultivar Manitoba were obtained from Early's Garden (SK, Canada). The plant cultivars within each plant species were selected based on the differences in plant phenology (e.g. tomato cultivar Manitoba plants had a higher number of visible trichomes compared to tomato cultivar Amish Paste and lettuce cultivar Parris Island Cos typically grew higher vertically compared to lettuce cultivar Winter Density). Seed was sanitized with a 2.7% sodium hypochlorite (Alfa Aesar, MA, USA) solution for 30 minutes and rinsed with sterile distilled water prior to germination at 4°C in the dark for three days on a moistened cotton pad (VWR International, PA, USA). After three days, single germinated seeds were transferred to the surface of 10% Murashige and Skoog Basal Medium (Sigma Aldrich, ON, Canada) supplemented with 1.2% agar (Difo, Becton Dickinson, NJ, USA) and 2% sucrose in five mL microcentrifuge tubes (Corning Axygen, NY, USA). Seedlings were grown in 72 cell seed starter trays fitted with clear plastic domes (GH-72, Jiffy Products of America Inc., OH, USA). Cotton pads soaked with 25 mL sterile distilled water were placed in each cell to generate a moist atmosphere inside the dome. The microcentrifuge tubes were then partially inserted into the cells, taking care to avoid contact between the pads and the surface of the propagation medium. The domed trays were held in a growth chamber set at 22 ± 1°C for 16 h with LED lights on and 16 ± 1°C for 8 h with LED lights off until the plants reached the four-leaf stage (3 weeks for both lettuce cultivars and tomato cultivar Manitoba, two weeks for tomato cultivar Amish Paste).

2.2.2 *Salmonella* inoculum preparation

The *Salmonella* strains (43) used in the study were from The *Salmonella* Foodborne Syst-OMICS Database (SALFOS, Université Laval, QC, Canada, https://salfos.ibis.ulaval.ca/). The strains, their serotypes and source of isolation are provided in Table 1. Twenty-six strains were from serovars that have been implicated in fresh produce outbreaks and seventeen from serovars seldom or never reported in such incidents. Each strain was grown in 10 mL of Tryptic Soy Broth (TSB) (Difco, Becton Dickinson, NJ, USA) overnight at 37°C with agitation (175 rpm). Ten mL of bacterial culture were then spun at 1811 x *g* for 10 min, the resulting pelleted cells were washed with ten mL 0.5 mM potassium phosphate buffer (PPB), pH 6.8-7.0 (Amresco, OH, USA), spun twice at 1811 x *g* for 10 min and re-suspended in ten mL 0.5 mM PPB. One mL of the suspension was adjusted spectrophotometrically to OD_{600} within the range 0.47-0.52. Inocula for experiments were prepared by mixing one mL of the latter with 999 mL 0.5 mM PPB with a stir bar in a 2 L beaker placed on a magnetic mixer operated at low speed at room temperature. This procedure yielded inocula containing 4-5 log colony forming units (CFU)/mL.

Table 1. List of Non-typhoidal *Salmonella* (NTS) strains used in this study

***Salmonella* strains**	**Specific source**
Agona S213*	Dried mussel
Agona S215*	Chill tank
Anatum S443	Environmental
Arizonae S172	Poultry
Bareilly S258	Human
Berta S333	Cheese

Canada S30	Chocolate
Chingola S32	Seaweed
Enteritidis S187*	Leafy greens
Enteritidis S3*	Human
Hadar S219	Chicken
Havana S286	Human
Heidelberg S191	Cocoa beans
Infantis S198	Greek pasta
Javiana S200*	Human
Javiana S203*	Octopus
Litchfield S272*	Human
Litchfield S273*	Shrimp
Liverpool S346	Cantaloupe
Luciana S43	Cantaloupe
Mbandaka S236*	Poultry
Mbandaka S238*	Pasta
Montevideo S239*	Thyme
Montevideo S241*	Poultry
Muenchen S206*	Cantaloupe
Muenchen S207*	Orange juice
Newport S195*	Alfalfa seed
Newport S2*	Human
Ohio S316	Goat

Oranienburg S216	Flour
Poona S306*	Beef roast
Poona S307*	Cantaloupe
Rubislaw S348	Horse
Saintpaul S204*	Sprouted chia seed
Saintpaul S205*	Shrimp
Senftenberg S269*	Alfalfa seed
Senftenberg S270*	Processed cheese
Thompson S193*	Spinach
Thompson S194*	Feather meal
Typhimurium S189*	Chocolate
Typhimurium S441*	Florida isolate, environmental
Uganda S276	Hog carcass
Uganda S277	Ground beef

*Strains are the top 13 most common serovars responsible for fresh produce outbreaks

2.2.3 Whole plant seedling inoculation and measurement of *Salmonella* populations

A device designed to receive six microcentrifuge tubes at a time (Figure 1) was used to inoculate whole plants. A total of 3-13 biological replicates were used depending on the *Salmonella* strain. One plant was used for each biological replicate. Aerial plants parts approximately 0.5 cm above the top of the microcentrifuge for lettuce cultivars and 3 cm above the top for tomato cultivars were immersed in the inoculum for 5 seconds. During the inoculation procedure, the magnetic stir plate was operated at the lowest speed setting at room temperature.

The inoculated plants were then held upside down in a biosafety cabinet until the leaves appeared dry. One half was returned to the cells in the seedling trays, taking care to avoid contact between the pads and the plants or between plants. The domes were returned to the trays which were then placed in an incubator at 21 ± 2°C. The remaining plants were cut at the base of the stem and placed in sterile 100 x 15mm petri dishes (VWR International, PA, USA) for measurement of weight. The weighed plants were then transferred to glass test tubes containing 9 mL 0.1% (w/v) peptone (Difco, Becton Dickinson, NJ, USA) which were agitated on a vortex for 30 seconds. Decimal dilutions were prepared in 0.1% peptone and suitable aliquots were spread on Xylose Lysine Deoxycholate Agar (XLD) (Oxoid, ThermoFisher Scientific, MA, USA) to estimate *Salmonella* populations. The same procedure was used to estimate *Salmonella* populations on plants after 5 days of incubation at 21ºC.

Figure 1. Device used to inoculate lettuce (cultivars Parris Island Cos and Winter Density) and tomato (cultivars Amish Paste and Manitoba) plants grown in 10% Murashige & Skoog Basal medium supplemented with 1.2% agar and 2% sucrose

2.2.4 Laser Scanning Confocal Microscopy (LSCM)

LSCM was performed according to methods described by Macarisin et al. (2012) with some modifications. After plants were grown according to section 2.2.1, they were inoculated following methods outlined in section 2.2.3 with *Salmonella* inocula prepared from section 2.2.2. Strains used for LSCM (*S.* Litchfield S273, *S.* Agona S213 and *S.* Saintpaul S204) were shown to actively colonize seedlings from both plant species based on results from Table 2. Leaves from inoculated plants were incubated for five days and were cut into 1 cm X 1 cm sections that were placed into the wells of Thermo Scientific Nunc Lab-Tek II Chambered Coverglass with a No. 1.5

borosilicate glass bottom (Thermo Scientific, ThermoFisher Scientific, MA, USA). The leaf sections were washed three times with Phosphate Buffered Saline (PBS) (VWR Life Science, VWR International, PA, USA) to remove unattached or loosely attached bacteria and fixed with -20°C methanol (BDH Chemicals, VWR International, PA, USA) for 5 min. After fixing, the sections were washed three times with PBS and incubated in PBS with non-fat dry milk (1:50 dilution; Biotium, CA, USA) for 30 minutes to block non-specific immunoglobulin binding in the subsequent steps. Following blocking, the sections were incubated for two hours in PBS-Tween 20 (Sigma-Aldrich, ON, Canada) with anti-*Salmonella* rabbit polyclonal antibody (FITC-fluorescein isothiocyanate) (1:50 dilution; ProSci Incorporated, CA, USA) and washed three times with PBS-Tween 20. Differential interference contrast and confocal fluorescence images were acquired simultaneously with a Fluotar VISIR 25x/0.95 NA and HC PL APO CS2 63x/1.20 NA water immersion objective of a Leica TCS SP8X LSCM system (Leica, Leica-Microsystems, France). Limits were set with a laser to 486 nm for sequential image acquisition and the HyD2 detector capture window was set between 498 nm and 526 nm. HyD4 detector capture window was set between 614 nm and 658 nm for fluorescein detection and laser set to 537 nm. 1024 X 1024-pixel resolution images were obtained with a Leica Application Suite LAS X 3.1.5.1.16308 software. Three-dimensional projections of the plant leaf surfaces (adaxial and abaxial) were constructed from the images using IMARIS x64 version 7.3.1 Software (Bitplane, Switzerland).

2.2.5 Statistical analysis

A range of 3 – 13 trials were conducted using independently grown plant crops and bacterial cultures. A range of 3 – 13 seedlings were analyzed at each sampling time interval. *Salmonella* populations were analyzed on $\log_{10}$-transformed data by one-way analysis of variance

(ANOVA) and Tukey's honestly significant difference (HSD) for means separation. All statistical analyses were performed using RStudio, version 1.1.453 (RStudio, Inc, MA, US).

2.3 Results and Discussion

2.3.1 Fate of different *Salmonella* strains on tomato and lettuce plant cultivars

The fate of 43 *Salmonella* strains from 29 serovars on seedlings from two tomato and lettuce cultivars was assessed using cultural methods. Preliminary experiments were carried out with two *Salmonella* Poona and Thompson strains to examine the time course of population growth on the plant seedlings and to select a time point for comparison (Appendix A). The growth curves showed that the *Salmonella* strains had reached the end of stationary phase after 5 days on both tomato and lettuce seedlings. Consequently, population measurements obtained immediately after inoculation (Day 0) and the change in population over 5 days (Day 5-0) were used for comparative purposes.

Population measurements at Day 0 and Day 5-0 shown in Table 2 were indicative of considerable variability in the behavior of the 43 *Salmonella* strains on the lettuce and tomato seedlings. For example, immediately after inoculation (Day 0), populations of *S.* Anatum S443 were significantly lower ($P < 0.05$) than the other strains on both lettuce cultivars but were significantly higher ($P < 0.05$) on tomato cultivar Manitoba (Table 2). After the five-day incubation, *S.* Anatum S443 populations decreased on both tomato cultivars but increased significantly ($P < 0.05$) by 2-3 log CFU/g on both lettuce cultivars. *S.* Arizonae S172 populations were also significantly ($P < 0.05$) lower on three of the plant cultivars and the lowest ($P < 0.05$) on lettuce cultivar Winter Density immediately after inoculation (Table 2). After five days, *S.* Arizonae S172

populations decreased or remained stagnant on both tomato cultivars and lettuce cultivar Parris Island Cos but increased 3.61 ± 0.49 log CFU/g on lettuce cultivar Winter Density (Table 2). High populations of strain *S.* Javiana S200 were also recovered from lettuce cultivars Winter Density and Parris Island Cos, and from tomato cultivar Amish Paste immediately after inoculation. Populations of this strain were also significantly higher ($P < 0.05$) on lettuce cultivars Parris Island Cos and tomato cultivar Manitoba (Table 2).

These results suggested that the interaction between *Salmonella* and growing plants is highly strain-specific. Previous studies on the ecology of *Salmonella* in the plant phyllosphere have primarily employed strains from serovars that are often implicated in produce-associated outbreaks (Shi et al. 2007; Brandl and Amundson 2008; Li and Uyttendaele 2018; Cui et al. 2018). Reasons for the disproportionate association of some serovars with outbreaks linked to fresh produce remain unclear, but it has been suggested that strains from other serovars may lack characteristics that favor the initial interaction with plants surfaces and the ability to survive and/or grow on the plant surface. In the present study several strains from such serovars (eg. *S.* Bareilly S258, *S.* Berta S333, *S.* Chingolo S32 and *S.* Rubislaw S348) were shown to persist on growing plants, and many were capable of growth on at least one of four plant cultivars. In contrast, some strains from serovars commonly implicated in produce-associated outbreaks displayed limited colonization potential on any of the plants (eg. *S.* Enteriditis S187, *S.* Typhimurium S189). These observations provided strong evidence that the ability to colonize the plants was strain- rather than serovar-specific.

Table 2. Populations (log CFU/g) of 43 *Salmonella* strains inoculated onto lettuce cultivar Parris Island Cos, lettuce cultivar Winter Density, tomato cultivar Amish Paste and tomato cultivar Manitoba plants immediately after inoculation (Day 0) and changes in population (Day 5-0) after 5 days of incubation at 21 ± 2°C.

Salmonella **serovar and strain**	**Plant Species and Cultivar**							
	Lettuce Parris cv. Island Cos		**Lettuce cv. Winter Density**		**Tomato cv. Amish Paste**		**Tomato cv. Manitoba**	
	Day 0[1]	**Day 5-0[2]**	**Day 0[3]**	**Day 5-0[4]**	**Day 0[5]**	**Day 5-0[6]**	**Day 0[7]**	**Day 5-0[8]**
Agona S213	2.61 ± 0.41^{abcd}	2.80 ± 0.63^{abcdefg}	2.80 ± 0.37^{abcde}	3.93 ± 1.01^{abcde}	3.09 ± 0.66^{abcdef}	3.10 ± 0.18^{abcde}	3.67 ± 0.29^{abcde}	1.97 ± 0.62^{abcdef}
Agona S215	1.81 ± 0.28^{d}	3.06 ± 0.39^{abcdef}	1.73 ± 0.27^{e}	3.71 ± 1.09^{bcdef}	2.31 ± 0.24^{efgh}	0.14 ± 0.56^{fghij}	2.09 ± 0.23^{f}	3.44 ± 0.79^{abcgh}
Anatum S443	2.39 ± 0.86^{abcd}	2.04 ± 1.56^{bcdefghi}	2.72 ± 1.10^{abcde}	2.91 ± 0.92^{cdefg}	3.30 ± 1.22^{abcdei}	-0.84 ± 0.52^{ij}	4.30 ± 0.71^{abg}	-2.12 ± 0.48^{i}
Arizonae S172	3.57 ± 0.40^{aefg}	0.07 ± 1.41^{j}	2.71 ± 1.10^{abcde}	3.61 ± 0.49^{bcdef}	3.92 ± 0.54^{abcijkl}	-0.51 ± 0.45^{hij}	4.79 ± 0.47^{ag}	-0.53 ± 0.83^{ij}
Bareilly S258	4.02 ± 0.33^{ef}	1.42 ± 1.41^{defghij}	3.82 ± 0.46^{afg}	3.00 ± 1.77^{cdefg}	4.84 ± 0.17^{jk}	0.12 ± 1.44^{fghij}	4.93 ± 0.48^{g}	2.53 ± 0.81^{abcdeh}
Berta S333	3.63 ± 0.46^{efg}	2.44 ± 0.39^{abcdefgh}	3.29 ± 0.33^{abcdfg}	3.99 ± 0.59^{abcde}	4.32 ± 0.63^{jkl}	1.62 ± 0.50^{defghkl}	4.48 ± 0.48^{abg}	2.94 ± 0.63^{abch}
Canada S30	2.58 ± 0.84^{abcd}	-0.03 ± 0.64^{j}	2.22 ± 0.31^{cde}	2.37 ± 0.54^{defgh}	3.94 ± 0.57^{abcijkl}	1.82 ± 0.78^{cdefgkl}	4.16 ± 0.70^{abcg}	1.92 ± 1.18^{abcdefk}
Chingola S32	3.77 ± 0.29^{efg}	3.11 ± 0.52^{abcde}	3.52 ± 0.55^{abcfg}	2.89 ± 0.66^{cdefg}	4.03 ± 0.67^{abijkl}	-0.36 ± 0.63^{ghij}	4.58 ± 0.53^{abg}	1.62 ± 0.57^{abcdefjk}
Enteritidis S187	nd	2.55 ± 0.54^{abcdefgh}	nd	1.14 ± 0.91^{ghi}	nd	2.94 ± 0.40^{abcde}	1.96 ± 0.09^{f}	2.64 ± 1.40^{abcdeh}
Enteritidis S3	4.46 ± 0.57^{e}	2.14 ± 0.61^{bcdefghi}	3.81 ± 0.15^{abfg}	2.53 ± 0.34^{defgh}	4.15 ± 0.43^{aijkl}	3.11 ± 0.60^{abcde}	4.12 ± 0.28^{abcg}	2.71 ± 0.62^{abcdh}
Hadar S219	2.30 ± 0.71^{bcd}	1.76 ± 0.48^{cdefghij}	2.59 ± 0.72^{bcde}	2.08 ± 0.69^{efgh}	2.96 ± 0.50^{abcdef}	0.81 ± 0.75^{fghil}	2.64 ± 0.67^{fhi}	0.38 ± 0.82^{efjk}
Havana S286	3.15 ± 0.75^{abcfg}	2.76 ± 0.19^{abcdefg}	3.37 ± 0.43^{abcdfg}	2.95 ± 1.17^{cdefg}	3.44 ± 0.47^{abcdeil}	1.47 ± 0.22^{defghkl}	4.18 ± 0.65^{abcg}	2.37 ± 0.34^{abcdeh}
Heidelberg S191	2.01 ± 0.23^{cd}	3.70 ± 0.42^{ab}	1.92 ± 0.18^{e}	$>1.92 \pm 0.18$	3.02 ± 1.03^{abcdefg}	2.20 ± 0.88^{bcdefkl}	4.02 ± 0.85^{abcdg}	2.21 ± 0.84^{abcdeh}
Infantis S198	3.09 ± 1.13^{abcfg}	2.96 ± 0.56^{abcdefg}	2.73 ± 0.89^{abcde}	3.90 ± 0.82^{abcdef}	3.85 ± 0.34^{abcdijkl}	1.75 ± 1.04^{cdefghkl}	3.83 ± 0.41^{abcdeg}	1.58 ± 1.12^{abcdefjk}
Javiana S200	3.79 ± 0.53^{efg}	2.93 ± 0.70^{abcdefg}	2.97 ± 0.84^{abcdeg}	3.89 ± 1.14^{bcdef}	2.43 ± 0.46^{efgh}	3.70 ± 0.62^{abc}	3.11 ± 0.53^{cdehi}	4.30 ± 0.69^{gh}
Javiana S203	2.74 ± 0.71^{abcdg}	2.23 ± 0.78^{bcdefghi}	3.68 ± 0.40^{abcfg}	3.12 ± 1.11^{cdefg}	2.37 ± 0.33^{efgh}	1.77 ± 0.72^{cdeghkl}	3.51 ± 0.53^{bcdeh}	2.10 ± 0.67^{abcdefh}
Litchfield S272	2.09 ± 0.17^{bcd}	0.12 ± 0.47^{ij}	1.89 ± 0.21^{e}	2.99 ± 0.45^{cdefg}	nd	3.30 ± 0.88^{abcde}	2.35 ± 0.59^{fi}	1.58 ± 0.52^{bcdefjk}
Litchfield S273	3.15 ± 0.70^{abfg}	3.42 ± 0.74^{abc}	2.21 ± 0.50^{de}	5.34 ± 0.70^{ab}	nd	4.13 ± 0.52^{am}	2.35 ± 0.59^{fi}	4.63 ± 1.38^{g}
Liverpool S346	2.75 ± 0.90^{abcdg}	2.66 ± 0.25^{abcdefg}	3.00 ± 1.27^{abcdefg}	2.52 ± 0.73^{defgh}	2.20 ± 0.56^{fgh}	2.37 ± 0.49^{abcdefkl}	4.64 ± 0.28^{abg}	1.75 ± 0.56^{abcdefjk}
Luciana S43	nd	0.72 ± 0.86^{ghij}	3.37 ± 1.15^{abcdfg}	1.25 ± 0.59^{fghi}	2.45 ± 0.88^{defgh}	1.31 ± 2.04^{efghkl}	4.40 ± 0.67^{abg}	0.70 ± 0.30^{defjk}

Mbandaka S236	1.84 ± 0.24^{d}	0.70 ± 0.73^{hij}	1.96 ± 0.09^{e}	2.50 ± 0.80^{defgh}	1.93 ± 0.15^{h}	2.56 ± 0.33^{abcdekl}	2.12 ± 0.22^{f}	1.97 ± 0.59^{abcdef}
Mbandaka S238	3.32 ± 0.90^{abefg}	2.84 ± 0.44^{abcdefg}	3.62 ± 0.25^{abcfg}	3.35 ± 1.23^{bcdefg}	2.19 ± 0.28^{fgh}	3.37 ± 0.48^{abcde}	2.58 ± 0.32^{fhi}	3.93 ± 0.85^{agh}
Montevideo S239	nd	2.24 ± 0.05^{bcdefghi}	nd	3.23 ± 0.89^{cdefg}	2.21 ± 0.49^{fgh}	3.56 ± 0.56^{abcd}	1.97 ± 0.35^{f}	4.26 ± 0.97^{gh}
Montevideo S241	nd	3.08 ± 0.81^{abcde}	nd	4.78 ± 1.45^{abc}	nd	2.94 ± 0.55^{abcde}	nd	3.94 ± 0.80^{gh}
Muenchen S206	2.26 ± 0.44^{bcd}	4.21 ± 0.67^{a}	2.23 ± 0.32^{cde}	4.45 ± 0.79^{abcd}	2.55 ± 0.48^{defgh}	4.25 ± 1.30^{am}	2.01 ± 0.19^{f}	2.88 ± 1.64^{abch}
Muenchen S207	2.03 ± 0.05^{cd}	4.41 ± 1.14^{a}	2.35 ± 0.31^{cde}	5.85 ± 0.21^{a}	2.36 ± 0.45^{efgh}	5.64 ± 0.91^{m}	1.89 ± 0.16^{f}	4.47 ± 1.02^{gh}
Newport S195	2.15 ± 0.13^{bcd}	2.95 ± 0.76^{abcdefg}	3.30 ± 0.73^{abcdfg}	2.90 ± 0.79^{cdefg}	3.83 ± 0.18^{abcdijkl}	2.77 ± 0.91^{abcde}	3.79 ± 0.24^{abcdeg}	2.07 ± 1.08^{abcdefh}
Newport S2	3.02 ± 0.42^{abcfg}	2.56 ± 1.02^{abcdefgh}	3.33 ± 0.27^{abcdfg}	3.01 ± 0.45^{cdefg}	4.01 ± 0.61^{abijkl}	2.68 ± 0.15^{abcdek}	4.39 ± 0.49^{abg}	1.35 ± 0.72^{cdefjk}
Ohio S316	1.89 ± 0.20^{d}	3.22 ± 0.41^{abcde}	2.39 ± 0.70^{cde}	2.41 ± 0.80^{defgh}	2.09 ± 0.57^{fgh}	2.45 ± 0.78^{abcdekl}	2.42 ± 0.65^{fi}	3.82 ± 1.17^{agh}
Oranienburg S216	nd	0.43 ± 0.66^{ij}	1.96 ± 0.10^{e}	-0.08 ± 0.21^{i}	2.09 ± 0.40^{fgh}	1.93 ± 0.65^{cdefkl}	2.31 ± 0.61^{fi}	2.77 ± 0.69^{abch}
Poona S306	nd	2.68 ± 0.23^{abcdefg}	nd	2.88 ± 1.61^{cdefg}	nd	4.52 ± 1.65^{am}	nd	nd
Poona S307	nd	3.01 ± 0.63^{abcdefg}	2.10 ± 0.23^{de}	1.07 ± 1.50^{ghi}	2.82 ± 0.70^{bcdefgh}	4.02 ± 1.20^{acbm}	nd	nd
Rubislaw S348	3.97 ± 0.34^{efg}	0.84 ± 1.43^{fghij}	3.90 ± 0.54^{afg}	1.62 ± 0.31^{efghi}	4.66 ± 0.18^{jkl}	-2.04 ± 0.62^{j}	4.91 ± 0.14^{g}	1.77 ± 0.15^{abcdefjk}
Saintpaul S204	4.32 ± 0.68^{e}	2.25 ± 0.69^{bcdefgh}	3.93 ± 0.48^{fg}	2.97 ± 0.85^{cdefg}	4.21 ± 0.32^{ijkl}	1.98 ± 0.52^{cdefkl}	4.72 ± 0.43^{ag}	2.97 ± 0.46^{abcgh}
Saintpaul S205	2.43 ± 0.90^{abcd}	2.57 ± 0.36^{abcdefgh}	2.13 ± 0.28^{de}	3.53 ± 1.04^{bcdef}	2.68 ± 0.40^{cdefgh}	3.65 ± 0.84^{abcd}	2.81 ± 0.61^{efhi}	2.58 ± 0.53^{abch}
Senftenberg S269	nd	1.01 ± 1.14^{fghij}	nd	2.45 ± 0.75^{defgh}	2.01 ± 0.02^{gh}	0.06 ± 0.19^{ghij}	1.94 ± 0.28^{f}	2.90 ± 0.57^{abch}
Senftenberg S270	2.86 ± 0.40^{abcdfg}	2.34 ± 1.17^{abcdefgh}	3.07 ± 0.19^{abcdefg}	2.72 ± 0.44^{cdefg}	3.61 ± 0.17^{abcdikl}	2.63 ± 0.73^{abcdekl}	3.84 ± 0.50^{abcdeg}	2.24 ± 0.61^{abcdeh}
Thompson S193	4.44 ± 0.23^{e}	1.35 ± 0.70^{efghij}	4.23 ± 0.27^{f}	2.39 ± 0.52^{defgh}	4.93 ± 0.30^{j}	1.37 ± 0.37^{efghkl}	4.87 ± 0.24^{g}	1.32 ± 0.67^{cdefjk}
Thompson S194	2.37 ± 0.36^{abcd}	3.25 ± 0.89^{abcd}	2.23 ± 0.53^{cde}	3.95 ± 0.37^{abcde}	1.92 ± 0.34^{fgh}	2.72 ± 0.72^{abcde}	2.43 ± 0.41^{fi}	3.48 ± 0.41^{abgh}
Typhimurium S189	2.32 ± 0.32^{abcd}	0.76 ± 0.75^{fghij}	2.04 ± 0.09^{de}	1.22 ± 0.95^{ghi}	2.00 ± 0.00^{gh}	1.00 ± 1.30^{fghkl}	1.93 ± 0.27^{f}	1.43 ± 0.27^{cdefjk}
Typhimurium S441	3.93 ± 0.64^{efg}	2.24 ± 0.31^{bcdefghi}	3.94 ± 0.43^{fg}	3.05 ± 0.69^{cdefg}	4.60 ± 0.28^{jkl}	1.71 ± 0.48^{defghkl}	4.93 ± 0.36^{g}	2.16 ± 0.18^{abcdeh}
Uganda S276	1.95 ± 0.11^{cd}	4.27 ± 0.95^{a}	2.23 ± 0.33^{cde}	3.33 ± 0.86^{cdefg}	2.31 ± 0.37^{efgh}	4.08 ± 0.48^{abm}	2.93 ± 0.48^{defhi}	2.54 ± 1.38^{abcdeh}
Uganda S277	2.11 ± 0.27^{bcd}	3.92 ± 0.76^{ab}	2.02 ± 0.19^{de}	3.81 ± 1.01^{bcdef}	2.88 ± 0.26^{bcdefgh}	3.34 ± 0.54^{abcde}	2.61 ± 0.56^{fhi}	3.76 ± 1.21^{abgh}

Means and standard deviations were calculated using data from n = ≥ 3 biological replicates.

nd: not detected at levels < 100 CFU/g

[1] Different superscripts (a-g) in the Lettuce Parris Island Cos Day 0 column read from top to bottom indicate significant differences between *Salmonella* strains immediately after inoculation on Lettuce Parris Island Cos

[2] Different superscripts (a-j) in the Lettuce Parris Island Cos Day 5-0 column read from top to bottom indicate significant differences between *Salmonella* strains after 5 days incubation on Lettuce Parris Island Cos

[3] Different superscripts (a-g) in the Lettuce Winter Density Day 0 column read from top to bottom indicate significant differences between *Salmonella* strains immediately after inoculation on Lettuce Winter Density

[4] Different superscripts (a-i) in the Lettuce Winter Density Day 5-0 column read from top to bottom indicate significant differences between *Salmonella* strains after 5 days incubation on Lettuce Winter Density

[5] Different superscripts (a-l) in the Tomato Amish Paste Day 0 column read from top to bottom indicate significant differences between *Salmonella* strains immediately after inoculation on Tomato Amish Paste

[6] Different superscripts (a-m) in the Tomato Amish Paste Day 5-0 column read from top to bottom indicate significant differences between *Salmonella* strains after 5 days incubation on Tomato Amish Paste

[7] Different superscripts (a-i) in the Tomato Manitoba Day 0 column read from top to bottom indicate significant differences between *Salmonella* strains immediately after inoculation on Tomato Manitoba

[8] Different superscripts (a-k) in the Tomato Manitoba Day 5-0 column read from top to bottom indicate significant differences between *Salmonella* strains after 5 days incubation on Tomato Manitoba

The data in Table 2 provided additional evidences that characteristics unique to individual strains rather than serovar dictated the behavior of *Salmonella* on plants. This was illustrated by differences observed with different strains of the same serovar. Populations of *S.* Typhimurium S189 were significantly ($P < 0.05$) lower than populations of *S.* Typhimurium S441 at both sampling intervals on all plant species and cultivars. Variable populations of *S.* Enteriditis S187 and S3, *S.* Litchfield S272 and S273, *S.* Mbandaka S236 and S238, *S.* Montevideo S241 and S206, *S.* Saintpaul S204 and S205, *S.* Senftenberg S269 and S270 and *S.* Typhimurium S189 and S441 were also recovered at Day 0 or after 5 days of incubation, although this was not consistent across plant species or cultivar, suggesting that additional factors intrinsic to plants species or cultivar thereof also influenced the fate of *Salmonella.* Indeed, data in Table 2 shows that populations of three strains (*S.* Agona S213, *S.* Berta S333 and *S.* Infantis S198) increased on both lettuce cultivars, while several others (eg. *S.* Arizonae S172, *S.* Canada S30, *S.* Litchfield S272, *S.* Heidelberg S191 and *S.* Mbandaka S236) only did so on one of the lettuce cultivars.

At first glance, visual attempts to correlate initial or post-inoculation populations of the 43 *Salmonella* strains with specific plant species or cultivars were unsuccessful. The lack of a correlation indicates that the interaction between plant host and colonizing *Salmonella* is likely complex and subject to several interacting factors. The influence of plant phenotypic characteristics on the ecology of bacterial pathogens such as *Salmonella* in the phyllosphere has not been examined in detail. It has been speculated that anatomical differences in plants from distinct species, or cultivars thereof, could induce considerable variability in surface features

available for attachment and colonization. Moreover, compositional differences in the outer cuticle of plants or the presence and activity of plant defense mechanisms may exert additional selective pressures after the initial interaction with the plant surface (Kerstiens 1996; Riederer and Schreiber 2001; Burton and Bhushan 2006; Jeffree 2006). Clearly, additional research is needed to define and characterize factors that influence the interaction between plant host and *Salmonella* that ultimately determine the fate of the species in the growing plant environment.

2.3.2 Sites colonized by *Salmonella* on tomato and lettuce leaf surfaces

LSCM images obtained after 5 days of incubation at 21ºC were provided in Figure 2. *Salmonella* cells were primarily located within stomata, in surface depressions adjacent to stomata or in random microsites not associated with specific anatomical features on tomato cultivar Manitoba and lettuce cultivar Parris Island Cos. In keeping with several previous reports on the interaction between *Salmonella* and plant leaves, both single cells and cellular aggregates suggestive of active colonization and growth were observed within these sites (Brandl and Amundson 2008; Barak et al. 2011). In contrast with the latter study and similar microscopic studies of leaf surfaces, trichomes were not apparent in any of the confocal images. Leaf trichome type, structure, chemical composition and density are known to vary between species, within cultivar of the same species and at various stages of plant maturity (Schilmiller et al. 2008). It must be noted that seedlings, rather than mature plants, were examined in the present work.

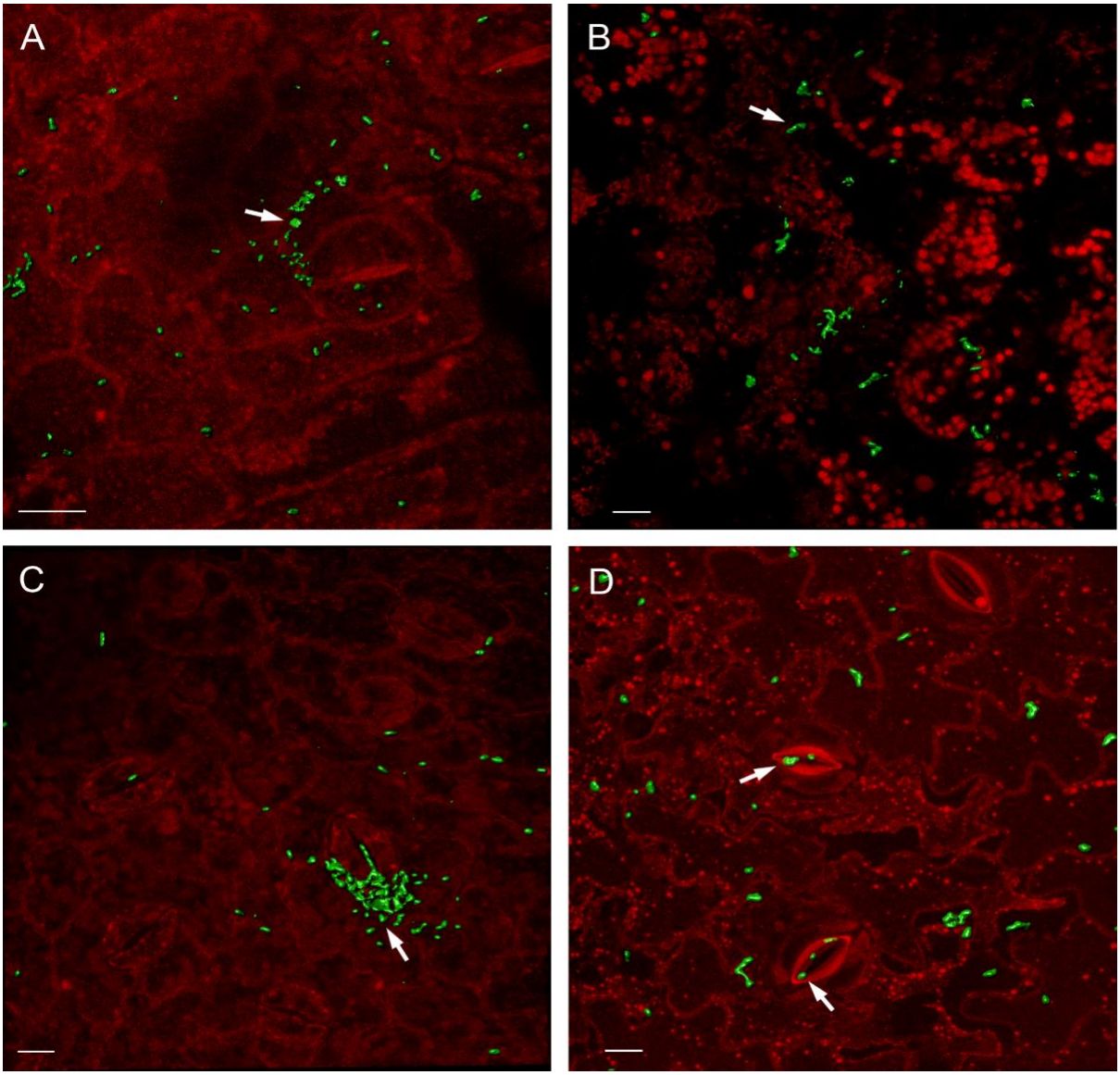

Figure 2. Laser scanning confocal microscopy (LSCM) micrographs of fluorescein isothiocyanate (FITC) stained *Salmonella* on tomato and lettuce seedling leaves after 5 days of incubation at 21°C (scale bar = 20μm). (A) Adaxial surface, Tomato cv. Manitoba inoculated with *S.* Litchfield S273. The arrow points to cellular aggregates in the surface depression adjacent to a stoma. (B) Adaxial surface, Tomato cv. Manitoba inoculated with *S.* Agona S213. The arrow points to a small cellular aggregate on an indeterminate leaf surface feature. (C) Adaxial surface, Tomato cv. Manitoba top leaf surface with *S.* Saintpaul S204. The arrow points

to a large cellular aggregate associated with a stoma. (D) Abaxial surface, Lettuce cv. Parris Island Cos inoculated with *S.* Saintpaul S204. The arrows point to bacterial cells associated with the guard cells of stomata.

2.4 Conclusions

Inoculation of different cultivars of lettuce and tomato plants with 43 strains drawn from a range of serovars showed that the fate of *Salmonella* is highly strain-specific and subject to complex interactions between the host and the bacterium. The results of this study highlighted the need for caution in the interpretation of findings from research on the ecology of *Salmonella* on growing food plants carried out with a limited number of strains. For example, Han and Micallef (2014) concluded that *S.* Typhimurium was able to colonize tomato plant leaves better than *S.* Newport on the basis of experiments carried out with a single strain from both serovars. This conclusion appears premature on the basis of the present work which pointed to divergence in the colonization potential of two strains of *S.* Typhimurium on tomato leaves. In the absence of more detailed understanding of the phenotypic characteristics of *Salmonella* and plant hosts that induce variability in colonization behavior, caution mandates care in the selection of strains and the design of ecological studies in growing food plants. Replicated experimentation with multiple, well-characterized strains applied to different cultivars of host plant species is highly advisable in light of the strain-specificity described uncovered in the present work.

Microscopic examination of lettuce and tomato seedlings did confirm findings from previous studies and show examples of *Salmonella* colonization sites to be within stomates, surface depressions near stomates and in random microsites. In addition, the results of this work served to

identify several *Salmonella* strains that successfully colonize food plants. Three were chosen as "targets" for experiments intended to demonstrate the potential value of bacteriophage for their control as described in Chapter 3.

Chapter 3. Control of *Salmonella* on Romaine lettuce leaves using bacteriophages

3.1 Introduction

The use of bacteriophage as a means to inactivate bacteria was proposed soon after their discovery in 1915 (Sillankorva et al. 2012). Advantages of using bacteriophages as antimicrobial agents in foods include high specificity, self-replication, lack of negative sensory effects and general non-toxicity to humans (Sillankorva et al. 2012; Moye et al. 2018). The high specificity of bacteriophages allows for the general microbiota to remain untouched, thus avoiding undesirable effects due to alterations in spoilage patterns or the inactivation of desirable microorganisms in the food system (Sillankorva et al. 2012). Bacteriophages are tasteless and their addition does not alter the sensory characteristics or attributes of the food, which is of concern to many food processors (Moye et al. 2018).

Lytic rather than lysogenic bacteriophages are used to formulate preparations for use in food systems because they are known to be non-toxic to humans (Sillankorva et al. 2012). There have been instances of bacteriophages carrying virulence genes and passing those genes to the bacterial host cell during the lysogenic bacteriophage replication cycle (Fortier and Sekulovic 2013). An example is the cholera epidemic (Miller 2003). It has been suggested that CTXΦ, a bacteriophage containing genes encoding for cholera toxin, may have transferred the genes to *Vibrio cholerae* through the lysogenic replication cycle, leading to a cholera epidemic (Miller 2003). *Vibrio cholerae* was not previously known to cause illnesses in humans with toxins (Miller 2003).

Previous research has shown that the infectivity of bacteriophages as well as their stability during storage depends on the composition of solutions used for stabilization and preservation. Adams (1949) found that calcium ion solutions provide the highest infectivity and stability for bacteriophage T5. Other solutions were less effective for the preservation of bacteriophage T5, which lost its activity when stored in phosphate buffer and was inactivated when stored in citrate solution (Adams 1949). Moreover, the infectivity of bacteriophage is known to decrease during time in storage due to interactions with solution components (Mylon et al. 2010). For example, Mylon et al. (2010) found that the MS2 bacteriophage aggregated and lost infectivity when it was stored in increasing concentrations of calcium chloride due to the neutralization of the negatively charged moieties on the surface of the bacteriophage. Given this risk, bacteriophage suspensions for use in food systems are prepared in solutions containing millimolar concentrations of calcium chloride and are used soon after preparation.

There have been few attempts to use bacteriophages for the control of *Salmonella* on leafy vegetables. Therefore, the objective of the experiments described in this chapter was to determine the efficacy of a bacteriophage cocktail against *Salmonella* on Romaine lettuce leaves.

3.2 Materials and Methods

3.2.1 *Salmonella* strains and inoculum preparation

The strains selected for the experiments, *S.* Saintpaul S204, *S.* Saintpaul S205, and *S.* Typhimurium S441 were previously shown to be efficient colonizers of lettuce seedlings (Chapter 2) and were strains that can be lysed by all 5 bacteriophages used in the cocktail described in section 3.2.2 below as opposed to other strains that cannot be lysed by all 5 bacteriophages. Inocula

were prepared from each strain separately, as described in the materials and methods section 2.2.2, with the following changes. The cell suspension was spectrophotometrically adjusted to OD_{600} within the range 0.47 – 0.52, and was serially diluted in 0.5mM potassium phosphate buffer to obtain an inoculum containing 5-6 log colony forming units (CFU)/mL.

3.2.2 Bacteriophage cocktail

A cocktail consisting of 5 broad host range bacteriophages was prepared in 1.0mM $CaCl_2$. The bacteriophages (Felix 01, HER20, Φ3, Φ6 and SE13 (Table 3)) were isolated from a variety of sources by collaborating laboratories. The cocktail was formulated to contain a titer of 10^9 – 10^{10} plaque forming units (PFU)/mL measured by adding 50µl of the bacteriophage cocktail to 450µl of tryptic soy broth + 1.0mM $CaCl_2$ in capped microcentrifuge tubes. Decimal dilutions of the bacteriophage cocktail were performed and 50µl of the target *Salmonella* strain (*S.* Saintpaul S204, *S.* Saintpaul S205 or *S.* Typhimurium S441) were added to each of the capped microcentrifuge tubes separately. The target strain was mixed with the bacteriophage cocktail and the mixture was incubated at 37°C for 30 min before it was plated on a tryptic soy agar + 1.0mM $CaCl_2$ plate. The plates were incubated at 37°C for 24 hours prior to counting the plaques.

Table 3. Bacteriophages used in this study

Bacteriophage	**Source**
Φ3	Pretreated sludge from a sewage treatment facility in Montreal
Φ6	Pretreated sludge from a sewage treatment facility in Montreal
Felix01	Felix d'Herelle virus collection at Laval University, Quebec City

HER19	Felix d'Herelle virus collection at Laval University, Quebec City
SE13	Water after first treatment from a sewage treatment facility in Vancouver

3.2.3 Bacteriophage application to inoculated Romaine lettuce

Romaine lettuce was purchased from a local grocery store in Coquitlam, British Columbia, Canada. Sections (2x2 cm) were cut from the leaves with a scalpel and were placed onto 4.25 cm diameter filter papers (VWR International, PA, USA) inside 60 mm x 15 mm petri dishes (VWR International, PA, USA). 100 µL of bacteriophage cocktail + 1.0mM $CaCl_2$ or the 1.0mM $CaCl_2$ solution alone were applied to the leaf sections and were spread evenly across the surface using a plastic spreader. Lettuce sections were set aside without bacteriophage cocktail + 1.0mM $CaCl_2$ or 1.0mM $CaCl_2$ solution as the control. All were placed in an incubator set at 4°C overnight to allow drying of the surface. The next day 100 µl of the *Salmonella* inoculum were applied to the surface of each lettuce section and spread evenly across the surface as before. Sterile distilled water (500µl) was added to the filter paper and 2/3 of all lettuce sections were placed in an incubator set at 21°C. *Salmonella* populations were measured on 1/3 of the lettuce sections to retrieve population estimates immediately after inoculation. *Salmonella* populations were measured on the other 2/3 of the lettuce sections 1 and 2 days after inoculation.

3.2.4 Measurement of *Salmonella* populations

The lettuce sections were placed in capped glass test tubes containing 9mL of 0.1% peptone and were agitated on a vortex mixer set at high speed for 1 minute. Decimal dilutions were prepared

in 0.1% peptone and the fluids were spread on XLD agar to estimate *Salmonella* populations per surface area (CFU/cm^2) of the lettuce leaf section after 24 hours of incubation at 37ºC.

3.2.5 Statistical analysis

Five trials were conducted using independently grown bacterial cultures. Five leaf sections were analyzed at each sampling time interval across the different treatments: (1) *Salmonella* alone, (2) *Salmonella* + 1.0mM calcium chloride and (3) *Salmonella* + 1.0mM calcium chloride + bacteriophage cocktail. *Salmonella* populations were analyzed on $\log_{10}$-transformed data by one-way analysis of variance (ANOVA) and Tukey's honestly significant difference (HSD) for means separation. All statistical analyses were performed using RStudio, version 1.1.453 (RStudio, Inc, MA, US).

3.3 Results and Discussion

3.3.1 Effect of a bacteriophage cocktail on *Salmonella* inoculated onto Romaine lettuce

A bacteriophage cocktail was applied to Romaine leaf sections 24 hours before their inoculation with three *Salmonella* strains. Populations of each strain measured immediately, 1 day and 2 days after inoculation on leaf sections treated with 1.0mM calcium chloride + bacteriophage cocktail, 1.0mM calcium chloride and without additional treatment (controls) are shown in Figure 3. The bacteriophage cocktail was applied before *Salmonella* inoculation because preliminary results (Appendix C) obtained with *Salmonella* Saintpaul S204 and Enteriditis S187 showed that the bacteriophage cocktail was not effective in reducing *Salmonella* populations if it was applied 24 hours after inoculation on plant leaves. In the case of *Salmonella* Enteritidis S187, *Salmonella*

populations were higher when the bacteriophage cocktail was applied 24 hours after inoculation (Appendix C, Figure C3 and C4). Reasons for the inability of the bacteriophage cocktail to reduce *Salmonella* populations when the treatment was applied 24 hours after inoculation are unclear. It is possible that many *Salmonella* cells were located in depressions of the plant leaf surface that were not reached by the bacteriophages. Moreover, bacteriophage adsorption to the plant surface may have further reduced the probability of collision with *Salmonella* (Sillankorva et al. 2012).

As expected on the basis of observations reported in Chapter 2, populations of each strain increased on the untreated lettuce leaf sections over two days of incubation. Treatment with the 1.0mM calcium chloride solution alone had no effect on populations of *S.* Saintpaul S205 and *S.* Typhimurium S441 measured at each sampling interval (Figure 3 B and C). In contrast, populations of *S.* Saintpaul 204 were significantly lower ($P<0.05$) immediately after inoculation and after one day of incubation but were not significantly different from the controls after 2 days (Figure 3 C). The decline in *S.* Saintpaul 204 population measured immediately after inoculation was unanticipated as concentrations of calcium chloride in the millimolar range were not expected to be antagonistic to *Salmonella.* Moreover, the rapid population increase subsequent to inoculation suggested that the calcium chloride concentrations used in this work did not affect growth. However, calcium bridges formed with free carboxyl groups of pectin chains are suspected of favouring the attachment of *Salmonella* to plant cells (Tan et al. 2016). Hence, the presence of free calcium ions may have strengthened the interaction between *S.* Saintpaul 204 and leaf surface components, thereby reducing the removal of cells during sample preparation resulting in lower population measurements at Day 0.

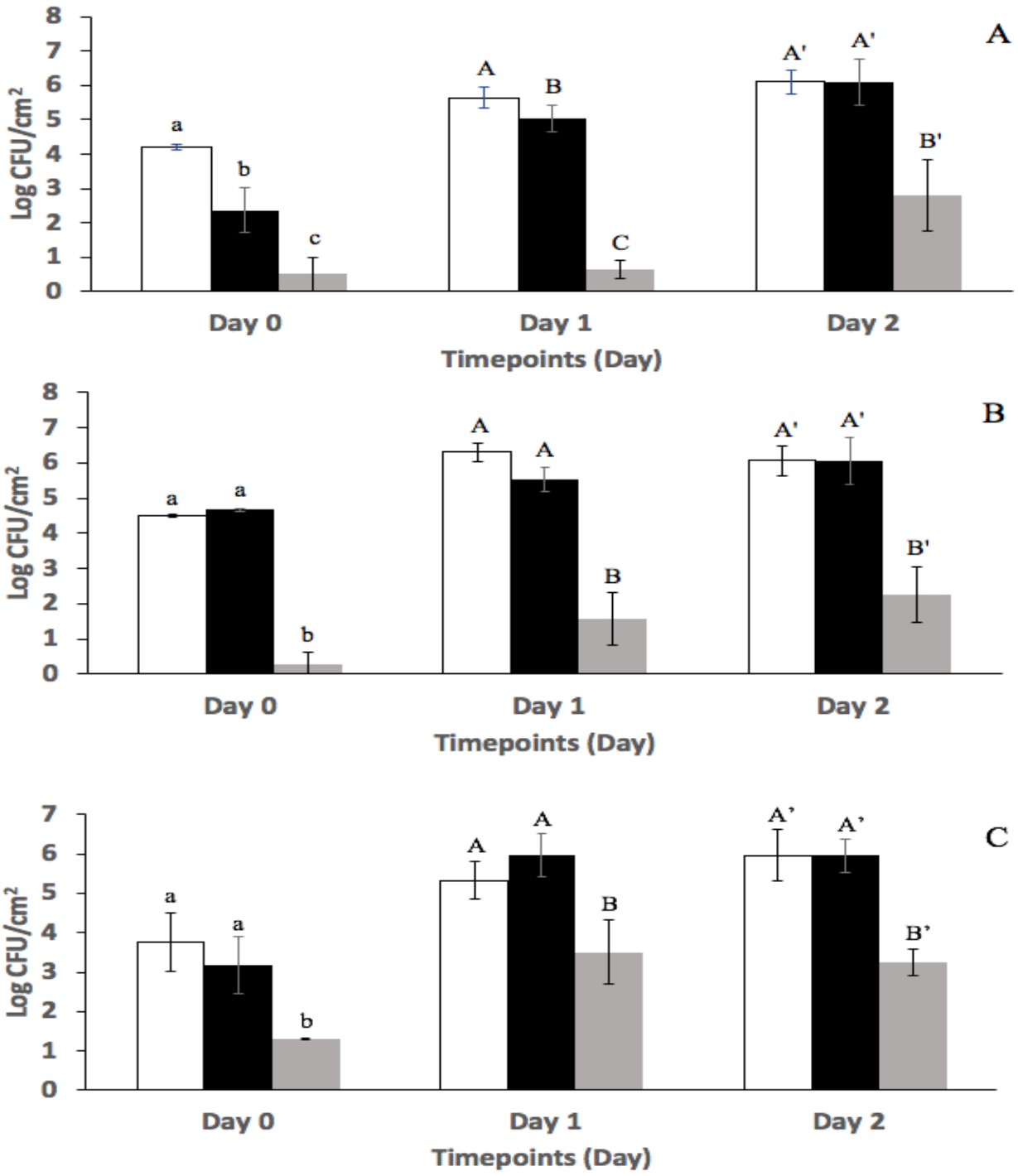

Figure 3. Populations (log CFU/cm^2) of *Salmonella* immediately (Day 0), 1 (Day 1) and 2 days (Day 2) after inoculation on Romaine lettuce leaf sections with different strains. Treatments: ☐: untreated lettuce leaf sections (controls); ■ : 1.0mM calcium chloride solution spread onto the

leaves 24 hours before inoculation; ■: 1.0mM calcium chloride + bacteriophage cocktail containing Felix01, HER19, Φ3, Φ6 and SE13 spread onto the leaves 24 hours before inoculation. (A) *Salmonella* Saintpaul S204. Different superscript (a-c) denotes significant differences ($P < 0.05$) between treatments on Day 0. Different superscript (A-C) denotes significant differences ($P < 0.05$) between treatments on Day 1. Different superscript (A'-B') denotes significant differences ($P < 0.05$) between treatments on Day 2. (B) *Salmonella* Saintpaul S205. Different superscript (a-b) denotes significant differences ($P < 0.05$) between treatments on Day 0. Different superscript (A-B) denotes significant differences ($P < 0.05$) between treatments on Day 1. Different superscript (A'-B') denotes significant differences ($P < 0.05$) between treatments on Day 2. (C) *Salmonella* Typhimurium S441. Different superscript (a-b) denotes significant differences ($P < 0.05$) between treatments on Day 0. Different superscript (A-B) denotes significant differences ($P < 0.05$) between treatments on Day 1. Different superscript (A'-B') denotes significant differences ($P < 0.05$) between treatments on Day 2. Mean and standard deviations were calculated using data from n = 5 biological replicates.

Application of the bacteriophage cocktail suspended in 1.0mM calcium chloride led to significant reductions in the populations of all three *Salmonella* strains on Romaine lettuce leaf sections. Immediately after inoculation, populations of *S.* Saintpaul S204 and *S.* Saintpaul S205 were reduced by 3.72 log CFU/cm^2 and 4.25 log CFU/cm^2 respectively (Figure 3 A and B) and populations of *S.* Typhimurium S441 by 2.47 log CFU/cm^2 (Figure 3 C). Moreover, differences between untreated controls and bacteriophage treated sections were sustained over one and two days of incubation. After two days, the magnitude of the difference was greatest for strain *S.* Saintpaul S205 (3.82 log CFU/cm^2) and lowest for *S.* Typhimurium S441 (2.72 log CFU/cm^2) (Figure 3 B and C). Susceptibility to specific bacteriophages is known to be highly strain-specific. Consequently, preparations for use in foods are typically composed of several bacteriophage in an effort to ensure consistent activity against a wide spectrum of strains of the target species (Chan et al. 2013). To this end, the cocktail used in the present work was formulated from 5 bacteriophages with broad host range against *Salmonella*. While the cocktail was clearly able to delay *Salmonella* population development on lettuce leaves, the effect was less pronounced with strain *S.* Typhimurium S441 than *S.* Saintpaul S204 and *S.* Saintpaul S205. This result indicated that differences in susceptibility are not limited to specific bacteriophage but may extend to cocktails prepared from several bacteriophages. In addition, the number of bacteriophages required to infect and lyse target bacteria (the multiplicity of infection) can also vary between strains. Consequently, differences in population reductions obtained with the 3 strains of *Salmonella* inoculated onto lettuce leaves may have been a consequence of variable susceptibility to infection dictated by the host range afforded by the bacteriophage cocktail, and/or the concentration of the bacteriophage cocktail applied to the leaf sections.

One of the anticipated benefits of bacteriophage-based food preservation is the ongoing release of infectious virion progeny to ensure continuous inhibition of target bacteria (Howard-Varona et al. 2017). Under this assumption, *Salmonella* populations should have declined or remained unchanged on lettuce leaf sections treated with the bacteriophage, which was clearly not the case. Several factors may impose limits on the efficacy of bacteriophages in food systems. Delivery to sites likely to be colonized by bacteria to favour contact between bacteriophage and target is a major challenge in the development of control strategies based on this approach. The bacteriophage cocktail used in the present work was distributed evenly in an attempt to saturate the lettuce leaf surface with bacteriophage. However, images provided in Chapter 2 (Figure 2 A and C) showed that *Salmonella* preferentially colonized sites that were located within surface depressions on the leaf surface, for example those adjacent to stomata. Given the hydrophobic nature of the leaf surface, it is possible that such sites are not readily accessible to aqueous bacteriophage solutions, resulting in insufficient delivery of infectious bacteriophage to locations where *Salmonella* populations were the highest. In addition, infectivity can be affected by chemical and physical stresses in the food environment, some of which can be foreseen (eg. pH extremes), but others unanticipated because they have not been characterized. Given the current dearth of information about micro-environments at the lettuce leaf surface, their influence on the fate of bacteriophage within such sites is unknown. In addition, bacteria are known to gain tolerance or outright resistance to bacteriophage, a process which can occur in very few bacterial generations (Gonçalves de Melo et al. 2018). The use of cocktails rather than single bacteriophage is meant to lessen the risks of resistance, but some bacteria have developed strategies that enable them to adapt quickly, particularly in environments where phage pressure is low (Abedon 2018). Hence, several factors can influence

bacteriophage infectivity, the rate at which virion progeny is released and overall efficacy against *Salmonella* on lettuce leaves.

3.4 Conclusions

Application of a bacteriophage cocktail prior to a challenge with three strains of *Salmonella* decreased *Salmonella* populations on Romaine lettuce leaves. The effect was strain-dependent despite the fact that the cocktail consisted of 5 broad host range bacteriophages.

Chapter 4. Conclusions and future research directions

4.1 Conclusions

Previous research carried out with a limited number of *Salmonella* strains hinted at strain-associated differences in capacity for attachment to plant surfaces, survival and/or growth on food plants (Shi et al. 2007; Berger et al. 2009; Kroupitski et al. 2009a; Kroupitski et al. 2009b; Barak et al. 2011). The first objective of the research was to determine the fate of a diverse collection of *Salmonella* strains isolated from a range of sources on growing lettuce and tomato plants. The results of the experimental trials carried out on seedlings from two cultivars of lettuce and tomato confirmed that plant colonization potential is highly variable within the different strains of *Salmonella*. Strains from serovars uncommonly associated with fresh produce outbreaks were shown to colonize growing plants as efficiently as strains from serovars that have rarely caused outbreaks. In the absence of more detailed understanding of the phenotypic and genotypic characteristics of *Salmonella* and plant hosts that induce variability in colonization behavior, caution mandates care in the selection of strains and the design of ecological studies in growing food plants. The use of *Salmonella* cocktails formulated with multiple, poorly characterized strains could introduce outlier biases should a single strain exhibits behavior(s) that are not characteristic of the species. Consequently, the results of this work strongly supported the notion that successful research on the ecology of *Salmonella* on growing food plants requires replicated experimentation with multiple, well-characterized strains applied to different cultivars of the host plant species grown under controlled environmental conditions.

The second objective of the research was to identify sites on growing lettuce and tomato plants that are actively colonized by *Salmonella*. Imaging of the plant surfaces by LSCM showed that cells were located within stomates, in surface depressions adjacent to stomates or in random microsites not associated with specific anatomical features, but there were no apparent differences in sites colonized by three *Salmonella* strains. Figure 2 images also provided evidence of active growth in the form of cellular aggregates in recessed locations, primarily near stomata, which was contrasted by the presence of numerous, randomly distributed single cells that did not appear to be dividing. Similar observations derived from previous research on the interaction between *Salmonella* and plant leaves have led other researchers to suggest that microenvironments within recessed locations provide conditions conducive to growth, particularly near stomates which are known to release water vapor and oxygen (Jones 1998). The prevailing opinion is that bacteria located within these microenvironments are metabolically active and shielded from harsh environmental stresses, including exposure to sanitizers during washing. However, the physiological state of surface-bound *Salmonella* cells at other random locations on the plant surface is uncertain and there is presently no experimental evidence that such cells are more sensitive to severe environmental stresses, including exposure to sanitizers. Moreover, the role of other behaviours associated with enhanced survival (for example, the viable-but-non-culturable state or persistence phenotype) on the efficacy of antimicrobial treatments applied for the control of *Salmonella* and other enteric bacterial pathogens in fresh produce remains largely unknown. Clearly, greater understanding of the physiological state of surface-bound cells located in sites that do not provide conditions conducive to growth is needed to guide the development of new strategies for the control of *Salmonella* and other enteric bacterial pathogens on food plants.

In the final objective of this research, a lytic bacteriophage-based strategy was investigated for the control of *Salmonella* on lettuce leaves. A cocktail comprised of 5 broad host range bacteriophages was shown to reduce, but not eliminate *Salmonella* on the surface of Romaine lettuce leaves. It should be noted that metabolically inactive bacterial cells resist infection by bacteriophage and that growth is required for lysis to occur. As mentioned above, there is no certainty that this is the case in all sites on the lettuce leaf surface that are colonized by *Salmonella.* Moreover, the efficacy of bacteriophage-based control relies on a high probability of collision between infectious bacteriophages and their hosts to initiate absorption and eventual lysis. Imaging of the leaf surface showed that sites colonized by *Salmonella* were separated by considerable distances. While the relative sparsity of targets at the outset was overcome by the application of high bacteriophage concentrations, the resumption of growth subsequent to the treatment was likely attributable to a reduced probability of collision with remaining *Salmonella* targets. Furthermore, bacteriophage titers tend to decline in foods due to adsorption by the matrix or decay caused by adverse chemical reactions. Hence the results of the research highlighted a key challenge in bacteriophage-based control of pathogens in foods, specifically difficulty in maintaining bacteriophage at titers that can ensure optimal destruction of bacterial targets over extended periods of time. Several strategies may be considered to overcome this problem, such as physical protection of the bacteriophage by encapsulation or repeated application to replenish titers. The latter approach could have proven useful in the present context where a second application may have curtailed further growth of *Salmonella* on Day 1 and 2. However, repeated applications may not be practical in commercial food systems and would engender additional costs.

4.2 Future Directions

Results of experiments described in Chapter 2 highlighted on-going knowledge gaps concerning the interaction between plant host and colonizing *Salmonella.* It remains unknown to what extent phenotypic traits associated with individual strains influence attachment to specific plant surface features or their ability to grow using nutrient resources available in the phyllosphere. Further research should be conducted to identify critical phenotypes associated with successful colonization of food plants. Moreover, comparative genomics of strains that exhibit different colonization behaviours would serve to identify relevant genetic features. Increased understanding of phenotypic and genetic traits responsible for colonization would support the development of methods to assess the risk implied by specific strains in food crops. The ability to accurately characterize risks to this level of precision is desirable given the increasing importance of fresh produce as a vehicle for the transmission of *Salmonella*.

While the present research in Chapter 3 showed that bacteriophage-based control of *Salmonella* has merit for fresh produce, successful applications will require strategies that maintain effective titers of bacteriophage at critical stages in the pre- to postharvest continuum. For example, washing systems could be exploited for the efficient delivery of bacteriophage to the plant surface but there would be a need to stabilize the phage preparations to prevent their deterioration due to mechanical action in processing systems. Alternatively, other antimicrobial strategies that work synergistically with bacteriophages could be considered (Moye et al. 2018).

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Appendices

Appendix A. Growth curves for 2 *Salmonella* serovars (*Salmonella* Thompson S193 & S194 and *Salmonella* Poona S306 & S307) conducted

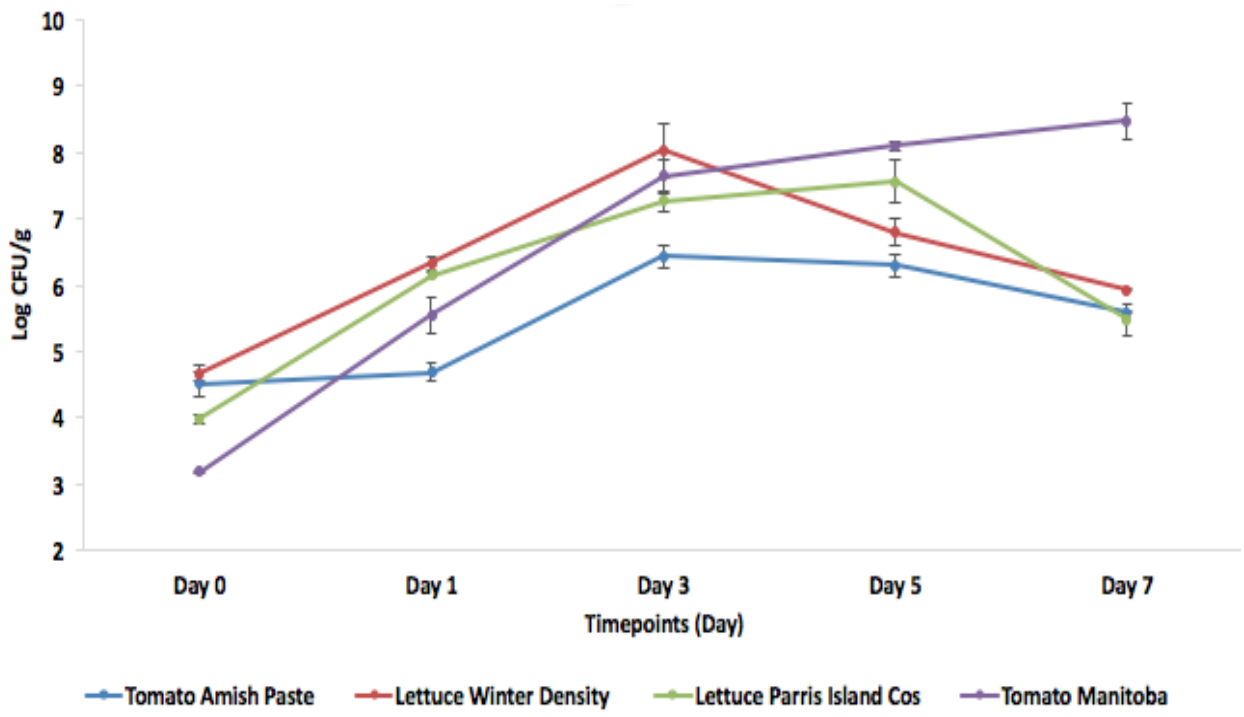

Figure A1. Populations of *Salmonella* Thompson S193 & S194 on tomato (cultivar Manitoba and Amish Paste) and lettuce (cultivar Winter Density and Parris Island Cos) plants after 0, 1, 3, 5 and 7 days at 21ºC. Each value represents the mean of 6 samples ± standard deviation

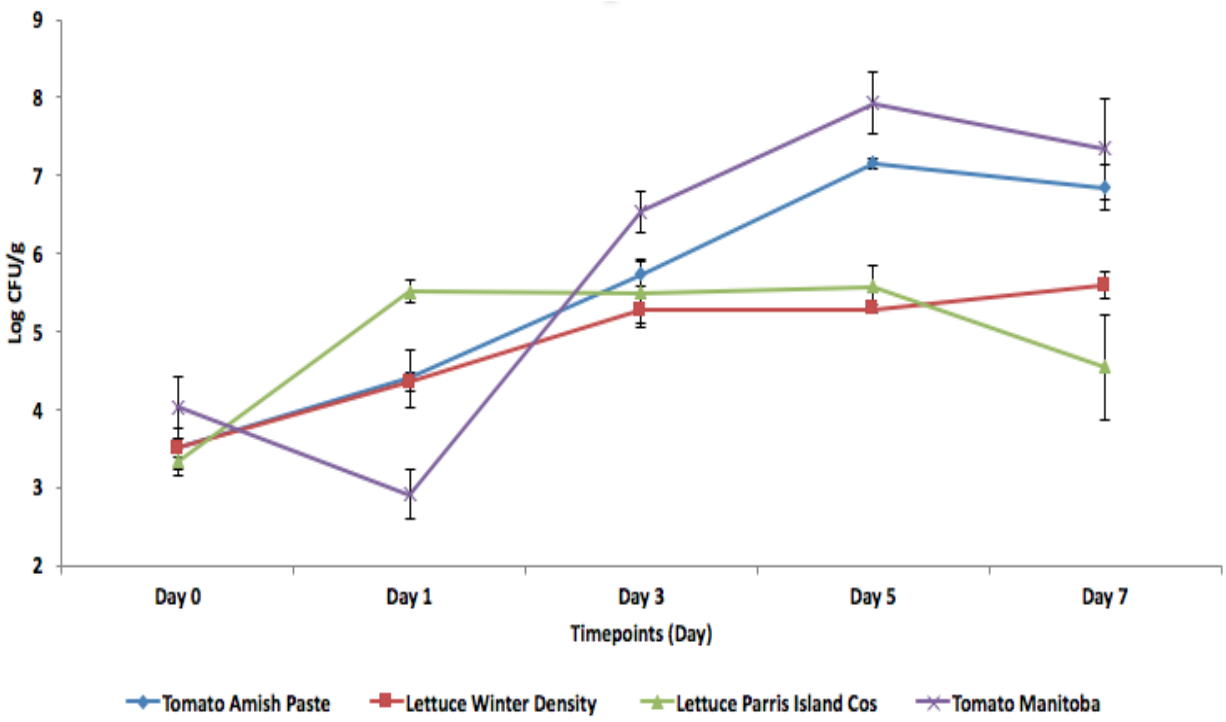

Figure A2. Populations of *Salmonella* Poona S306 & S307 on tomato (cultivar Manitoba and Amish Paste) and lettuce (cultivar Winter Density and Parris Island Cos) plants after 0, 1, 3, 5 and 7 days at 21ºC. Each value represents the mean of 6 samples ± standard deviation.

Appendix B. One-way ANOVA statistics between 43 *Salmonella* strains inoculated on 4 plant cultivars on day 0 and day 5-0

Table B1. One-way ANOVA between 43 *Salmonella* strains inoculated on lettuce (cultivar Parris Island Cos) on day 0

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
strain	42	163.95	3.904	14.13	<2e-16 ***
Residuals	220	60.79	0.276		

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Table B2. One-way ANOVA between 43 *Salmonella* strains inoculated on lettuce (cultivar Parris Island Cos) on day 5-0

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
strain	42	302.2	7.196	11.29	<2e-16 ***
Residuals	190	121.1	0.637		

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Table B3. One-way ANOVA between 43 *Salmonella* strains inoculated on lettuce (cultivar Winter Density) on day 0

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
strain	42	128.86	3.0681	10.16	<2e-16 ***
Residuals	219	66.12	0.3019		

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Table B4. One-way ANOVA between 43 *Salmonella* strains inoculated on lettuce (cultivar Winter Density) on day 5-0

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
strain	42	359.3	8.554	10.79	<2e-16 ***
Residuals	194	153.8	0.793		

Signif. codes: 0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

Table B5. One-way ANOVA between 43 *Salmonella* strains inoculated on tomato (cultivar Amish Paste) on day 0

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
strain	42	225.45	5.368	21.24	<2e-16 ***
Residuals	215	54.34	0.253		

Signif. codes: 0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

Table B6. One-way ANOVA between 43 *Salmonella* strains inoculated on tomato (cultivar Amish Paste) on day 5-0

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
strain	42	514.2	12.242	19.69	<2e-16 ***
Residuals	183	113.8	0.622		

Signif. codes: 0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

Table B7. One-way ANOVA between 43 *Salmonella* strains inoculated on tomato (cultivar Manitoba) on day 0

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
strain	42	291.80	6.948	32.69	<2e-16 ***
Residuals	208	44.21	0.213		

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Table B8. One-way ANOVA between 43 *Salmonella* strains inoculated on tomato (cultivar Manitoba) on day 5-0

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
strain	42	393.3	9.364	14.39	<2e-16 ***
Residuals	163	106.1	0.651		

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Appendix C. Growth curves for 2 *Salmonella* strains after bacteriophage treatment on lettuce (cultivar Parris Island Cos) and tomato (cultivar Manitoba) after 0, 3 and 5 days

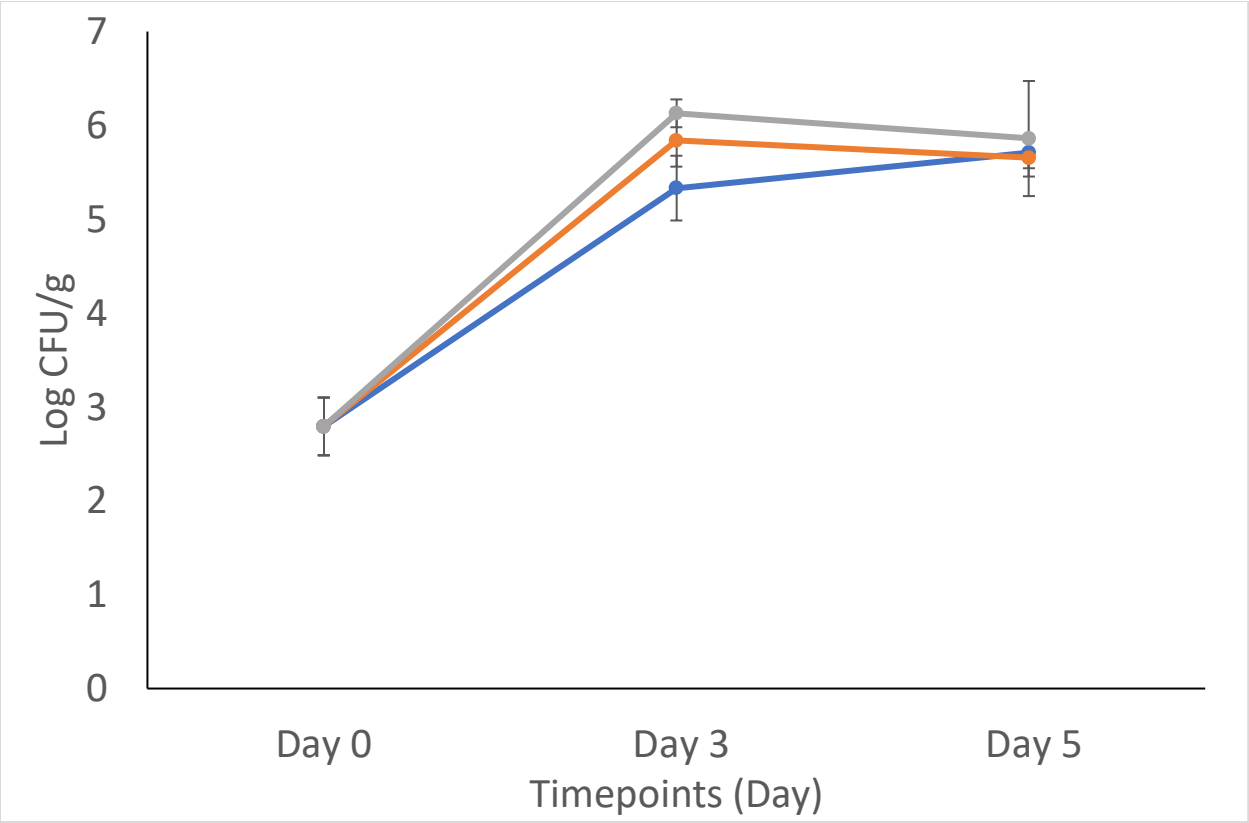

Figure C1. Populations (log CFU/g) of *Salmonella* Saintpaul S204 on lettuce (cultivar Parris Island Cos) plants immediately (Day 0), 3 (Day 3) and 5 days (Day 5) after inoculation at 21ºC. Treatments: : untreated lettuce plant leaves (controls); : 1.0mM calcium chloride solution spread onto the plant leaves 24 hours after inoculation; : 1.0mM calcium chloride + bacteriophage cocktail containing Felix01, HER19, Φ3, Φ6 and SE13 spread onto the leaves 24 hours after inoculation. Each value represents the mean of 5 biological replicates ± standard deviation.

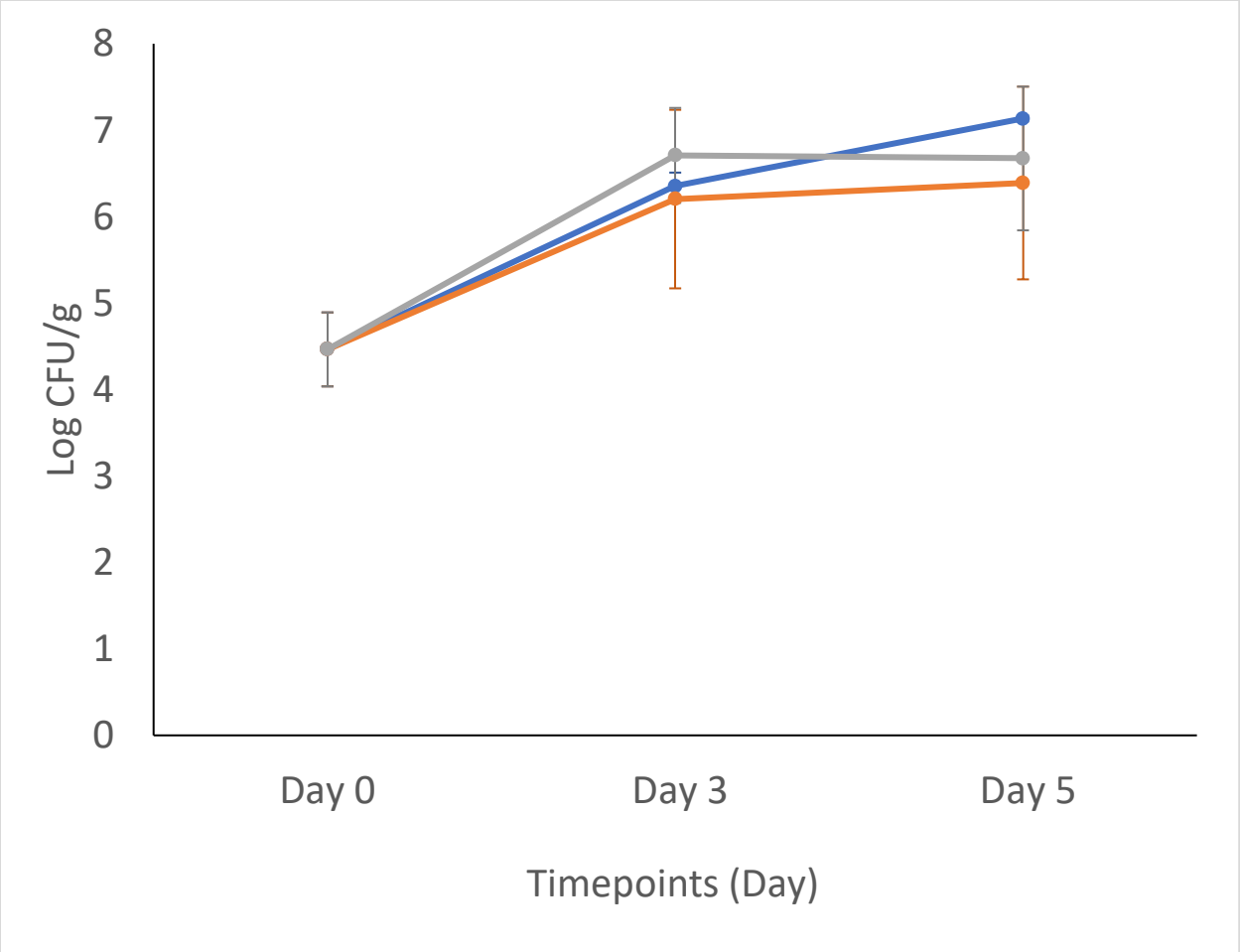

Figure C2. Populations (log CFU/g) of *Salmonella* Saintpaul S204 on tomato (cultivar Manitoba) plants immediately (Day 0), 3 (Day 3) and 5 days (Day 5) after inoculation at 21ºC. Treatments: : untreated tomato plant leaves (controls); : 1.0mM calcium chloride solution spread onto the plant leaves 24 hours after inoculation; : 1.0mM calcium chloride + bacteriophage cocktail containing Felix01, HER19, Φ3, Φ6 and SE13 spread onto the leaves 24 hours after inoculation. Each value represents the mean of 5 biological replicates ± standard deviation.

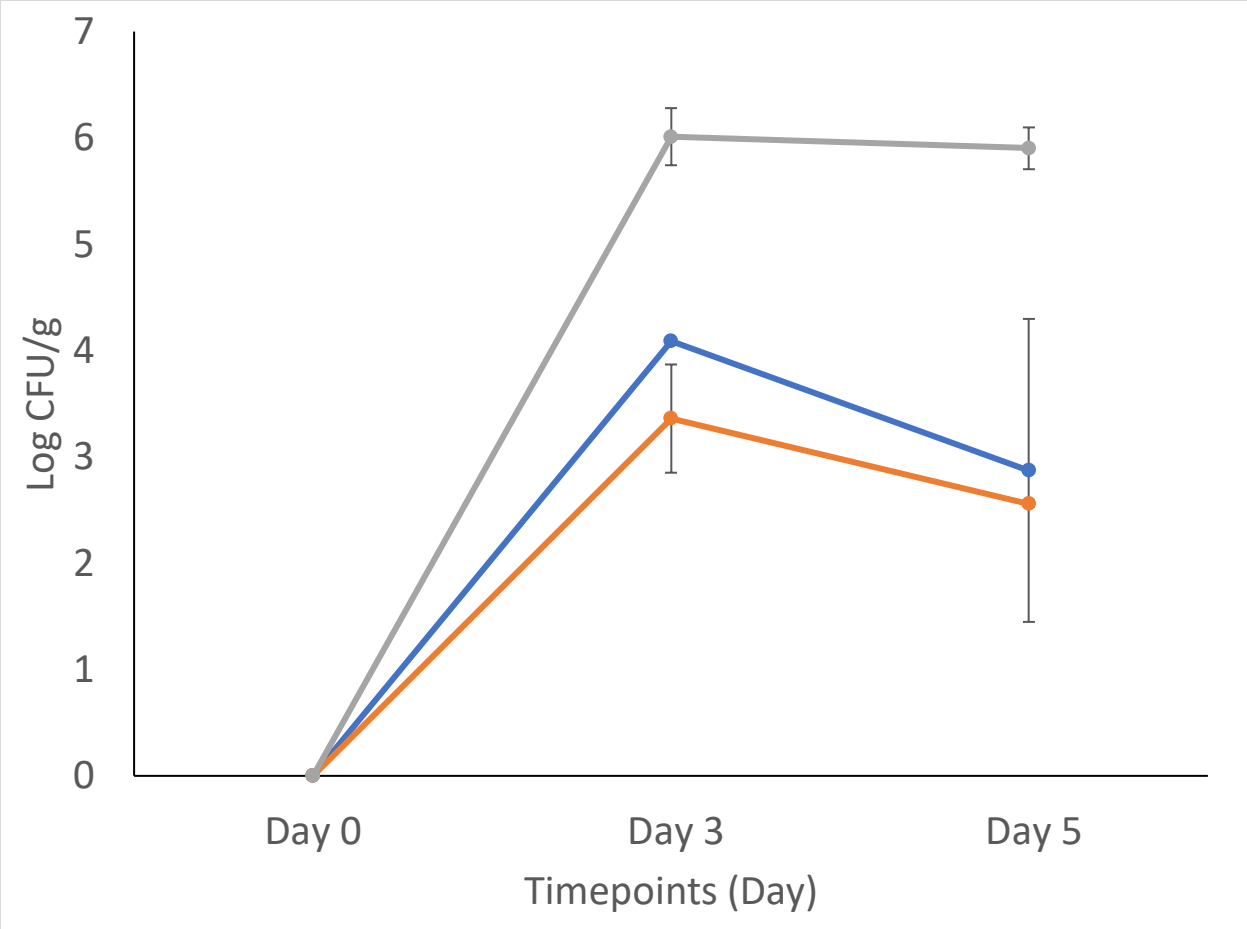

Figure C3. Populations (log CFU/g) of *Salmonella* Enteritidis S187 on lettuce (cultivar Parris Island Cos) plants immediately (Day 0), 3 (Day 3) and 5 days (Day 5) after inoculation at 21ºC. Treatments: : untreated lettuce plant leaves (controls); : 1.0mM calcium chloride solution spread onto the plant leaves 24 hours after inoculation; : 1.0mM calcium chloride + bacteriophage cocktail containing Felix01, HER19, Φ3, Φ6 and SE13 spread onto the leaves 24 hours after inoculation. Each value represents the mean of 5 biological replicates ± standard deviation.

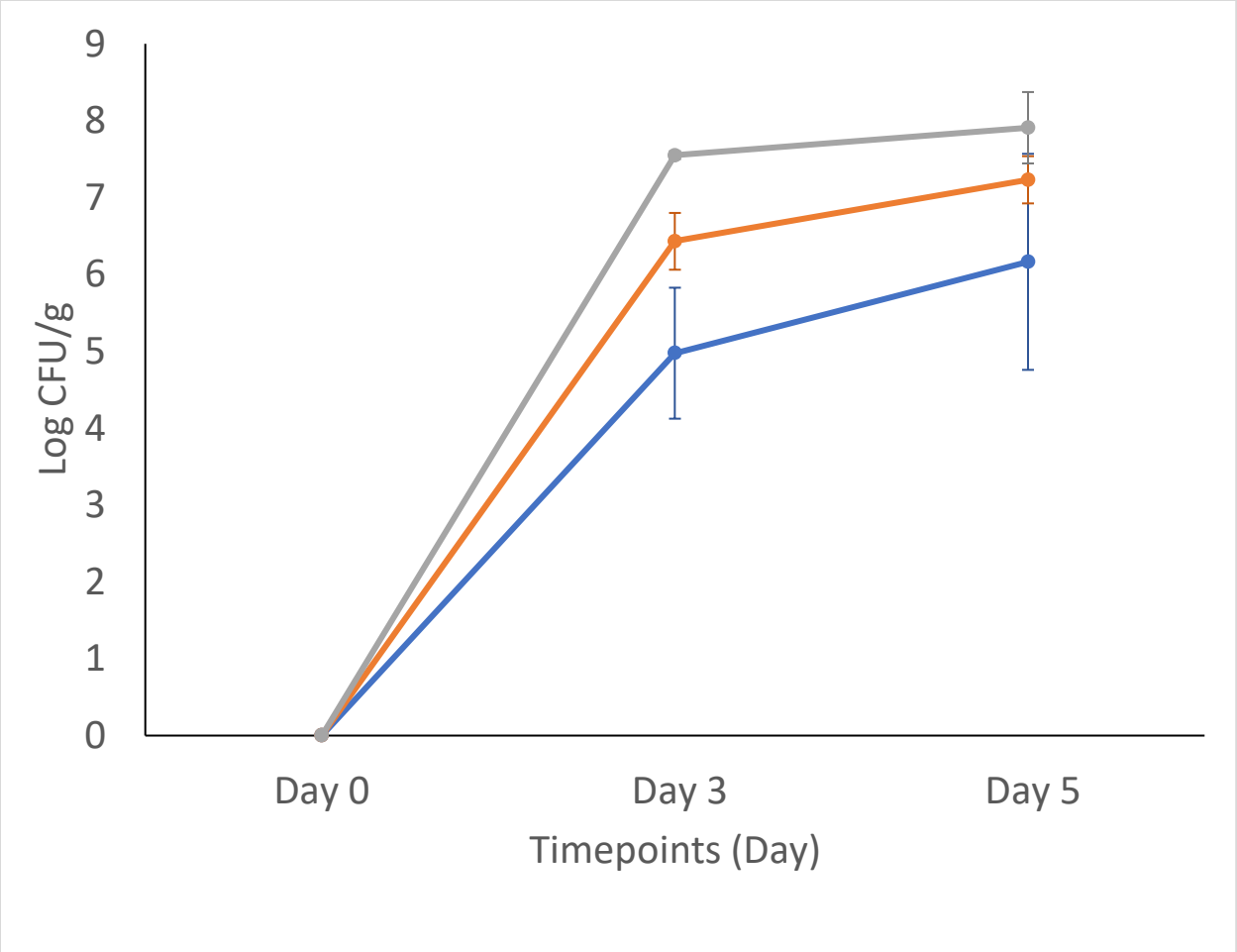

Figure C4. Populations (log CFU/g) of *Salmonella* Enteritidis S187 on tomato (cultivar Manitoba plants immediately (Day 0), 3 (Day 3) and 5 days (Day 5) after inoculation at 21ºC. Treatments: : untreated tomato plant leaves (controls); : 1.0mM calcium chloride solution spread onto the plant leaves 24 hours after inoculation; : 1.0mM calcium chloride + bacteriophage cocktail containing Felix01, HER19, Φ3, Φ6 and SE13 spread onto the leaves 24 hours after inoculation. Each value represents the mean of 5 biological replicates ± standard deviation.

Appendix D. One-way ANOVA and Tukey's HSD statistics for 3 *Salmonella* strains on day 0, day 1 and day 2

Table D1. One-way ANOVA between 3 treatments: (1) *S.* Saintpaul S204 strain, (2) *S.* Saintpaul S204 strain + 1.0mM $CaCl_2$, (3) *S.* Saintpaul S204 strain + 1.0mM $CaCl_2$ + bacteriophage at day 0

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
saint204d0t	2	34.69	17.345	86.13	1.91e-07 ***
Residuals	11	2.22	0.201		

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Table D2. Tukey's HSD for one-way ANOVA between 3 treatments: (1) *S.* Saintpaul S204 strain, (2) *S.* Saintpaul S204 strain + 1.0mM $CaCl_2$, (3) *S.* Saintpaul S204 strain + 1.0mM $CaCl_2$ + bacteriophage at day 0

	saint204d0t	std	r	Min	Max
chloride wash	2.3658606	0.65409784	4	1.57403127	3.051153
Inoculation	4.2342447	0.07676556	5	4.15381486	4.322219
phage	0.5091594	0.47650337	5	0.05115252	1.165096

Alpha: 0.05; DF Error: 11
Critical Value of Studentized Range: 3.819588

Treatments with the same letter are not significantly different.

	saint204d0t	groups
Inoculation	4.2342447	a
chloride wash	2.3658606	b
phage	0.5091594	c

Table D3. One-way ANOVA between 3 treatments: (1) *S.* Saintpaul S204 strain, (2) *S.* Saintpaul S204 strain + 1.0mM $CaCl_2$, (3) *S.* Saintpaul S204 strain + 1.0mM $CaCl_2$ + bacteriophage at day 1

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
saint204d1t	2	52.18	26.091	230.6	4.3e-09 ***
Residuals	10	1.13	0.113		

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Table D4. Tukey's HSD for one-way ANOVA between 3 treatments: (1) *S.* Saintpaul S204 strain, (2) *S.* Saintpaul S204 strain + 1.0mM $CaCl_2$, (3) *S.* Saintpaul S204 strain + 1.0mM $CaCl_2$ + bacteriophage at day 1

	saint204d1t	std	r	Min	Max
chloride wash	5.0472535	0.3895665	5	4.4851889	5.5471591
Inoculation	5.6483039	0.3064884	5	5.2941906	6.0133640
phage	0.6338819	0.2725486	3	0.3521825	0.8962506

Alpha: 0.05; DF Error: 10
Critical Value of Studentized Range: 3.876777

Treatments with the same letter are not significantly different.

	saint204d1t	groups
Inoculation	5.6483039	a
chloride wash	5.0472535	b
phage	0.6338819	c

Table D5. One-way ANOVA between 3 treatments: (1) *S.* Saintpaul S204 strain, (2) *S.* Saintpaul S204 strain + 1.0mM $CaCl_2$, (3) *S.* Saintpaul S204 strain + 1.0mM $CaCl_2$ + bacteriophage at day 2

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
saint204d2t	2	19.29	9.646	20.97	0.00041 ***
Residuals	9	4.14	0.460		

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Table D6. Tukey's HSD for one-way ANOVA between 3 treatments: (1) *S.* Saintpaul S204 strain, (2) *S.* Saintpaul S204 strain + 1.0mM $CaCl_2$, (3) *S.* Saintpaul S204 strain + 1.0mM $CaCl_2$ + bacteriophage at day 2

	saint204d2t	std	r	Min	Max
chloride wash	5.152497	0.6540438	5	4.306425	5.841203
Inoculation	6.099726	0.3420436	4	5.653213	6.482516
phage	2.800827	1.0192608	3	2.059753	3.963197

Alpha: 0.05; DF Error: 9

Critical Value of Studentized Range: 3.948492

Treatments with the same letter are not significantly different.

	saint204d2t	groups
Inoculation	6.099726	a
chloride wash	5.152497	a
phage	2.800827	b

Table D7. One-way ANOVA between 3 treatments: (1) *S.* Saintpaul S205 strain, (2) *S.* Saintpaul S205 strain + 1.0mM $CaCl_2$, (3) *S.* Saintpaul S205 strain + 1.0mM $CaCl_2$ + bacteriophage at day 0

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
saint205d0t	2	57.52	28.758	602.8	3.77e-11 ***
Residuals	10	0.48	0.048		

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Table D8. Tukey's HSD for one-way ANOVA between 3 treatments: (1) *S.* Saintpaul S205 strain, (2) *S.* Saintpaul S205 strain + 1.0mM $CaCl_2$, (3) *S.* Saintpaul S205 strain + 1.0mM $CaCl_2$ + bacteriophage at day 0

	saint205d0t	std	r	Min	Max
chloride wash	4.6613032	0.05537816	4	4.60138088	4.7107518
Inoculation	4.5165661	0.03938714	4	4.46890092	4.5563025
phage	0.2669888	0.34029840	5	0.05115252	0.8293038

Alpha: 0.05; DF Error: 10
Critical Value of Studentized Range: 3.876777

Treatments with the same letter are not significantly different.

	saint205d0t	groups
chloride wash	4.6613032	a
Inoculation	4.5165661	a
phage	0.2669888	b

Table D9. One-way ANOVA between 3 treatments: (1) *S.* Saintpaul S205 strain, (2) *S.* Saintpaul S205 strain + 1.0mM $CaCl_2$, (3) *S.* Saintpaul S205 strain + 1.0mM $CaCl_2$ + bacteriophage at day 1

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
saint205d1t	2	55.18	27.591	127.9	2.42e-08 ***
Residuals	11	2.37	0.216		

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Table D10. Tukey's HSD for one-way ANOVA between 3 treatments: (1) *S.* Saintpaul S205 strain, (2) *S.* Saintpaul S205 strain + 1.0mM $CaCl_2$, (3) *S.* Saintpaul S205 strain + 1.0mM $CaCl_2$ + bacteriophage at day 1

	saint205d1	std	r	Min	Max
chloride wash	5.521088	0.3383248	5	5.0133640	5.822005
Inoculation	6.298288	0.2740174	5	6.0723418	6.774061
phage	1.575483	0.7335824	4	0.6532125	2.263340

Alpha: 0.05; DF Error: 11
Critical Value of Studentized Range: 3.819588

Treatments with the same letter are not significantly different.

	saint205d1	groups
Inoculation	6.298288	a
chloride wash	5.521088	a
phage	1.575483	b

Table D11. One-way ANOVA between 3 treatments: (1) *S.* Saintpaul S205 strain, (2) *S.* Saintpaul S205 strain + 1.0mM $CaCl_2$, (3) *S.* Saintpaul S205 strain + 1.0mM $CaCl_2$ + bacteriophage at day 2

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
saint205d2t	2	45.65	22.826	54.25	9.76e-07 ***
Residuals	12	5.05	0.421		

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Table D12. Tukey's HSD for one-way ANOVA between 3 treatments: (1) *S.* Saintpaul S205 strain, (2) *S.* Saintpaul S205 strain + 1.0mM $CaCl_2$, (3) *S.* Saintpaul S205 strain + 1.0mM $CaCl_2$ + bacteriophage at day 2

	saint205d2	std	r	Min	Max
chloride wash	5.828404	0.6725537	5	5.165096	6.565257
Inoculation	6.066293	0.4204633	5	5.516039	6.403335
phage	2.252308	0.7957388	5	1.281601	3.021189

Alpha: 0.05; DF Error: 12

Critical Value of Studentized Range: 3.772929

Treatments with the same letter are not significantly different.

	saint205d2	groups
inoculation	6.066293	a
chloride wash	5.828404	a
phage	2.252308	b

Table D13. One-way ANOVA between 3 treatments: (1) *S*. Typhimurium S441 strain, (2) *S*. Typhimurium S441 strain + 1.0mM $CaCl_2$, (3) *Salmonella* strain + 1.0mM $CaCl_2$ + bacteriophage at day 0

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
typ441d0t	2	16.668	8.334	13.66	0.000809 ***
Residuals	12	7.323	0.610		

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Table D14. Tukey's HSD for one-way ANOVA between 3 treatments: (1) *S*. Typhimurium S441 strain, (2) *S*. Typhimurium S441 strain + 1.0mM $CaCl_2$, (3) *Salmonella* strain + 1.0mM $CaCl_2$ + bacteriophage at day 0

	typ441d0	std	r	Min	Max
chloride wash	3.175971	1.13935699	5	2.051153	4.563036
Inoculation	3.764637	0.72964206	5	2.916454	4.533073
phage	1.293056	0.01830693	5	1.273001	1.306425

Alpha: 0.05; DF Error: 12
Critical Value of Studentized Range: 3.772929

Treatments with the same letter are not significantly different.

	typ441d0	groups
Inoculation	3.764637	a
chloride wash	3.175971	a
phage	1.293056	b

Table D15. One-way ANOVA between 3 treatments: (1) *S.* Typhimurium S441 strain, (2) *S.* Typhimurium S441 strain + 1.0mM $CaCl_2$, (3) *Salmonella* strain + 1.0mM $CaCl_2$ + bacteriophage at day 1

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
typ441d1t	2	13.866	6.933	16.89	0.000897 ***
Residuals	9	3.693	0.410		

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Table D16. Tukey's HSD for one-way ANOVA between 3 treatments: (1) *S.* Typhimurium S441 strain, (2) *S.* Typhimurium S441 strain + 1.0mM $CaCl_2$, (3) *S.* Typhimurium S441 strain + 1.0mM $CaCl_2$ + bacteriophage at day 1

	typ441d1	std	r	Min	Max
chloride wash	5.964604	0.5617517	5	5.202420	6.665698
Inoculation	5.328037	0.4689538	3	4.790197	5.651399
phage	3.508917	0.8146866	4	2.477121	4.341187

Alpha: 0.05; DF Error: 9
Critical Value of Studentized Range: 3.948492

Treatments with the same letter are not significantly different.

	typ441d1	groups
chloride wash	5.964604	a
Inoculation	5.328037	a
phage	3.508917	b

Table D17. One-way ANOVA between 3 treatments: (1) *S.* Typhimurium S441 strain, (2) *S.* Typhimurium S441 strain + 1.0mM $CaCl_2$, (3) *Salmonella* strain + 1.0mM $CaCl_2$ + bacteriophage at day 2

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
typ441d12t	2	17.355	8.677	32.12	4.44e-05 ***
Residuals	10	2.702	0.270		

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Table D18. Tukey's HSD for one-way ANOVA between 3 treatments: (1) *S.* Typhimurium S441 strain, (2) *S.* Typhimurium S441 strain + 1.0mM $CaCl_2$, (3) *S.* Typhimurium S441 strain + 1.0mM $CaCl_2$ + bacteriophage at day 2

	typ441d2	std	r	Min	Max
chloride wash	6.013476	0.4258146	5	5.259773	6.285838
Inoculation	5.958014	0.6596733	5	5.079181	6.687975
phage	3.244031	0.3434361	3	2.852785	3.495718

Alpha: 0.05; DF Error: 10

Critical Value of Studentized Range: 3.876777

Treatments with the same letter are not significantly different.

	typ441d2	groups
chloride wash	6.013476	a
Inoculation	5.958014	a
phage	3.244031	b