Remediation of soil contaminated by *Salmonella enterica* to expedite plant or replant of vegetables

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Statement of authorship

First, I declare that this thesis is my *bona fide* work and all sources of materials used for it have been duly acknowledged. This thesis is submitted to the University of Sydney in fulfillment of the requirements for the Doctor of Philosophy. I solemnly declare that this thesis has not been submitted to any other institution anywhere for the award of any academic degree, diploma, or certificate. Brief quotations from this thesis are allowable without special permission provided that an accurate acknowledgement of the source is made. In all other instances, however, permission must be obtained from the author.

As specified in Chapter 5, diagrams, data and statistical analysis were provided by Dr Kim-Yen Phan-Thien. This constituted less than 1% of information presented in this thesis.

Mulatua Hailu

Abstract

The consumption of fresh produce has increased across the world because of health benefits associated with these products. At the same time, the rate of foodborne illnesses caused by the consumption of fresh produce, especially leafy greens which are mostly consumed raw, continues to be of concern. There are many sources of contaminants during the preharvest stages of vegetable production and processing. However, the use of improperly composted manure is a primary source of contamination both in conventional and organic production systems. Animal manure, particularly chicken manure, is the main environmental source of many enteropathogens in Australia. Of these, *Salmonella enterica* is found to be the main bacterial pathogen causing disease outbreaks associated with the consumption of fresh produce. In addition, *L. monocytogenes* is recognised as a soil resident, prevalent coloniser of decaying vegetation in agricultural systems and was considered as a second concern of bacteria in the present study.

The present study was conducted to investigate methods for remediating *Salmonella* contaminated soil with the following objectives: (1) to assess the effect of environmental factors and soil type on the persistence and survival of *Salmonella* serovars under controlled conditions, (2) to determine the potential of low-residue cover crops to enhance die-off of *S. enterica* in contrasting soils in Australia, (3) to determine if single or combined cover crop-solarisation treatment facilitate die-off of *S. enterica* in soil so that there is no contamination associated with the re-planting of leafy greens, and (4) to assess the prevalence of *Listeria monocytogenes* in cover crop-amended soils in natural field conditions.

In the first part of this study a controlled microcosm pot trial was performed where four different *Salmonella* serovars were incubated at three temperatures (5, 21 and 37°C) under two moisture regimes (constant and fluctuating) and using two soil types (classified as clay loam and coarse sand; referred to as ‘clay’ and ‘sandy’ soils, respectively) with or without manure. Sampling was done every week and extraction and enumeration of *Salmonella* from the soil samples followed standard cultural methods. Because of the large number of treatments, only two-way interactions between the factors – manure, soil type, temperature, moisture, serovars and time – were considered. When there was a significant interaction between the factors, Tukeys honest significant difference (HSD) test was performed for multiple comparison tests. Most of the interactions analysed were significant (P <0.05).

Graph has been included to show the proportion of positive or negative *Listeria* cells after enrichment since the data from *Listeria* study was not statistically significant. Generally, the survival of *Salmonella* was found to be lower in sandy soil than clay soil, lower at higher temperatures than at lower temperatures, greater under constant moisture than fluctuating moisture regimes and greater in the presence of manure than in the absence of manure.

In the field study, a field site (Karalee Farm, sandy soil and Pye Farm, clay soil) was deliberately inoculated with *Salmonella* using chicken manure as the inoculum carrier and treated with three cover crops and/or soil solarisation (using black plastic film) to remediate the experimentally contaminated soil. The field experiment followed a split-plot design based on three cover crops and control treatments. One split-plot was a control without solarisation, while the second split-plot was solarisation with black plastic after incorporating the cover crop treatments, or during the time of cover crop sowing for the control plots. Soil sampling was done on weekly basis at both sites. In addition, soil temperature, moisture, air temperature, and precipitation data were measured throughout the course of the field experiment. Soil sample extraction, plating, enumeration and enrichment techniques were similar to the pot study. The *Salmonella* count data were log-transformed and analysed as described above. The whole-plot, split-plot and sampling time were considered as fixed effects, whereas the block was considered as a random effect in the model. Overall, *Salmonella* declined from 4.56 $\log_{10}$ CFU g^{-1} on the day of chicken manure application to below the quantitative limit of detection (LOD; 1.18 $\log_{10}$ CFU g^{-1}) in the control (fallow-open) treatment 105 days later, showing the natural die off of the pathogen. *Salmonella* counts in the sandy soil were below the LOD 2 weeks after inoculation. In regards to the treatments used to suppress *Salmonella* in the soil, there was no significant difference among either the cover crop (grown for 4 weeks) or cover crop-solarisation (applied for 3 weeks) treatments in hastening the decline of *Salmonella*. However, the application of black plastic, alone, significantly influenced the decline of *Salmonella* in the field. The level of *Salmonella* fell below the LOD after day 56 in the fallow-solarisation treatment whereas, in the fallow-open treatment, the level fell below the LOD after 91 days.

Generally, *Salmonella* survival was better in clay soil than in sandy soil in both experiments, which indicated that longer (>90 days) exclusion periods between the application of untreated manure and crop harvest is required in clay soil. In addition, the presence of manure enhanced survival of *Salmonella* in both soil types (sandy and clay); however the effect of manure was more pronounced in the clay soil. Higher temperatures (>37°C) and fluctuating

moisture levels led to a faster decline of *Salmonella* in soil, with or without manure amendment compared with constant moisture levels. From this result it may be concluded that black plastic can be used as a solarisation treatment to remediate *Salmonella*-contaminated soil. Further research is required to exploit the potential of cover crops for their biocidal activity in suppressing *Salmonella* in the field.

This thesis is dedicated to my mum who has been the source of my inspiration

“It always seems impossible until it’s done”

Nelson Mandela

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List of abbreviations

BPW – buffered peptone water

CFU – colony forming unit

CM – chicken manure

CSP – cold shock protein

Cont – control

DF – degrees of freedom

EC – electrical conductivity

GFP – green fluorescent protein

GSL/s – glucosinolate/s

HSD – honest significant difference test

ITC/s – isothiocyanate/s

LOD – limit of detection

NA – nutrient agar

ppm – parts per million

SAR – structure activity relationship

SEM – scanning electron microscopy

Std – standard

T – temperature

TSA – trypticase soy agar

TSARP – rifampicin-amended trypticase soy agar

TSB – tryptic soy broth

TTB – tetrathionate broth base

UV – ultraviolet

UV-C – ultraviolet in C spectrum

XLD – xylose lysine deoxycholate

XLDRP – rifampicin-amended xylose lysine deoxycholate

XLT4 – xylose lysine tergitol 4

1. General introduction

The consumption of fruit and vegetables is widely promoted for health and wellbeing (Berger *et al.* 2010). However, uncooked fruit and vegetables have been implicated in a myriad of foodborne outbreaks and as a source of foodborne pathogens that have been traditionally associated with foods of animal origin (Sivapalasingam *et al.* 2004; Arthur *et al.* 2007; Berger *et al.* 2010). Of highest concern are leafy green vegetables such as lettuce, spinach, cabbage and salad leaves of all varieties since they potentially harbor microorganisms that cause foodborne illness (Dobhal *et al.* 2014; Mritunjay and Kumar 2015).

Bacterial pathogens are a major contributor to fresh produce-associated foodborne illnesses with *Escherichia coli* O157:H7, *Salmonella enterica* and *Listeria monocytogenes* the most common pathogens causing outbreaks (Park *et al.* 2012; Bradford *et al.* 2013). Between 1973–1997 bacteria were responsible for 60% of outbreaks in produce-associated incidences in the USA (Sivapalasingam *et al.* 2004) with *Salmonella* accounting for nearly half of the outbreaks. In the present study, *Salmonella* is the main focus of study as it was found to be the most common pathogen accounting for 53% of outbreaks in the USA, 50% in the European Union (Callejón *et al.* 2015). In Australia it is the second most notified cause of foodborne illness (OzFoodNet Working Group 2012a). In particular, numerous incidences of *Salmonella* outbreaks in Australia have occurred in fresh products such as cantaloupes (rockmelons), tomatoes and sprouts (OzFoodNet Working Group 2012).

There has been increasing public interest concerning the safety of foods and environmental impacts on food production. This is especially so for organic foods, which are grown from crops fertilised only with organic fertilisers or compost, and not chemical fertilisers. While non-organic production of leafy greens has moved away from use of organic amendments, public interest in consuming organic products has accelerated the use of compost even in vegetable crops that are usually consumed raw or without heat processing (Anonymous 2015b). Compost made from animal waste and other organic refuse can serve as a valuable nutrient resource for agricultural fields and decrease the environmental effects of inorganic fertiliser if they are treated properly (Gong *et al.* 2005). In Australia, the total chicken litter production is estimated to be over 1 million tonnes per annum and this litter is used in production of horticulture, pasture, turf and viticulture crops (Anonymous 2015a).

Utilisation of farm-yard manure is a cost-effective and environmenally friendly way to improve soil quality and to provide nutrients for plant growth (Semenov 2008). Worldwide many horticultural industries typically use animal manures as an organic fertiliser (Franz and van Bruggen 2008). In addition, poultry manure is known to contain a higher percentage of nitrogen and other elements than cattle manure (Gamliel and Stapleton 1997). Unfortunately a drawback is that untreated manure has the potential to contain bacteria that cause foodborne illness, including the serious enterohemorrhagic bacteria such as *E. coli* O157:H7 (Semenov 2008).

Contamination of leafy green vegetables can occur during field production from soil, manure, compost and irrigation water containing pathogenic contaminants (Natvig *et al.* 2002; Solomon *et al.* 2002; Islam *et al.* 2004c). This is most likely to occur after application of organic amendments such as manure to production sites, and from irrigation with water containing faecal contaminants (Franz and van Bruggen 2008). Worldwide, many *Salmonella* outbreaks involving fresh fruit and vegetables have been associated with from soil treated with contaminated manure (Abd-Elall and Maysa 2015). In addition, soil amendments based on untreated chicken manure have been recognised as potential contributors of foodborne pathogens in Australia (Chinivasagam 2010).

Listeria monocytogenes is recognised as a soil resident and is a prevalent coloniser of decaying vegetation in agricultural systems and, as such, transfer to fresh produce has become an important concern (Hoelzer *et al.* 2012). This organism is known to have longer survival under adverse environmental conditions than other bacterial pathogens that are important in causing foodborne illnesses (Fenlon 1999).

Contamination risk of vegetable crops grown in manure-amended soils depends primarily on the ability of pathogens to survive during crop production (Franz and van Bruggen 2008). *Salmonella* has been reported to survive up to 300 days in soil amended with manure (Garcia *et al.* 2010). *Salmonella* spp. in particular, are adept at withstanding and adapting to severe environments (Foster and Spector 1995; Arthurson *et al.* 2011) with some strains persisting in the environment for years, withstanding periods of stress and nutrient depletion (Gorski *et al.* 2011). A range of factors have been found to influence the survival of *Salmonella* in soil. Of these, temperature (Ongeng *et al.* 2015), moisture (Entry *et al.* 2000; Mubiru *et al.* 2000), manure amendment (Whipps *et al.* 2008), soil type (van Veen *et al.* 1997; Danyluk *et al.*

2008; Franz and van Bruggen 2008), interaction and possible antagonism with other soil microorganisms and protozoans (Garcia *et al.* 2010) are the most important.

To minimise pathogen load on fresh produce, different postharvest sanitising methods have been employed. However, many of the methods may not be effective because of the characteristics of the product, including the surface which influences microbial attachment. Other factors influencing sanitiser efficacy are the length of contact time and potential presence of biofilms on the produce surface (Franz and van Bruggen 2008). Industry is focused on intervention strategies to reduce the risk of contamination and combat the recent rise in foodborne disease associated with the eating uncooked fruit and vegetables (Semenov 2008). Therefore, it is necessary to find an effective way to inactivate faecal pathogenic bacteria in soil to ensure the biosafety of the soil and the produce grown in it (Wu *et al.* 2009).

For the purpose of this investigation, a collaborative partnership was established between the University of Sydney (Australia) and the University of California, Davis (USA) to develop remediation and recovery measures for suppressing *Salmonella* in contaminated soil following chicken manure application to the soil under both Australian and USA conditions. The measures used for evaluation of effective treatments included solarisation and biofumigation using phenolic- and glucosinolate-producing cover crops.

Solarisation using plastic film over soil amended with chicken manure has been found to reduce populations of soil-borne pathogens (Barbour 2002). It is a cost-effective and sustainable option when viewed as a replacement or supplement to biocidal chemical treatment such as methyl bromide, and has the added advantage of weed suppression. In addition, studies have shown that isothiocyanates (hydrolytic products of glucosinolates) from cover crops exhibit biocidal activity against microrganisms including fungi and bacteria as well as insects and other pests (Wilson *et al.* 2013).

Currently, there are no validated remediation strategies that growers could implement to reduce or eliminate the presence of naturally-occurring human pathogens in soil. In addition, there has been little research conducted on allelopatic biochemical plant products and the combination of solarisation and biofumigation in enhancing the die-off of *Salmonella* spp.and other pathogenic microorganisms in the soil. The present study investigated practical remediation measures (applied to soil contaminated by improper use of chicken manure) to minimise the survival of *S. enterica* and prevent the transfer of pathogens to the edible

portions of a harvested crop. The targeted remediation treatments included cover crops (‘Terranova’ oilseed radish, ‘Cappuccino’ Ethiopian mustard, ‘Fumig8tor’ sorghum) and soil solarisation (using black plastic), alone and in combination, to supress *S. enterica* in soils under conditions typical of Australian production regions. The aims of this research were to:

1. Assess the effect of environmental factors and soil types on the persistence and survival of *Salmonella* serovars under controlled conditions.
2. Determine the optimal low-residue cover crop containing glucosinolates or phenolic compounds that enhance die-off of *S. enterica* in contrasting soils in Australia.
3. Determine which single or combined cover crop-solarisation treatments facilitate die-off of *S. enterica* in soil so that there is no re-contamination associated with the re-planting of leafy greens produce.
4. Assess the presence of *Listeria monocytogenes* in cover crop-amended soils in natural field conditions.

The overall hypothesis for this study was:

Single or sequential strategies involving short-duration, low-residue cover crops and solarisation will be effective in the practical elimination of residual *Salmonella enterica* contamination.

2. Literature review

2.1 Safety of fresh produce

Fresh produce is known to confer substantial health and nutritional benefits and their consumption is rising (Abadias *et al.* 2008). However, fruit and vegetables are now recognised as common agents for the transfer of human pathogens from animal sources (Franz and van Bruggen 2008; Hanning *et al.* 2009; Berger *et al.* 2010). It is now well accepted that the consumption of fruit and vegetables is a risk factor for infection by enteric pathogens (Heaton and Jones 2008) such as bacteria, parasites and viruses, and a number of reports refer to raw vegetables harbouring a potential for foodborne pathogens (Abadias *et al.* 2008; Olaimat and Holley 2012; Allen *et al.* 2013; Goodburn and Wallace 2013). Food safety in fresh produce has now become a global issue given the large volumes of fresh produce that are traded nationally and internationally (Ongeng *et al.* 2015).

In the USA, 1990–2004, the number of cases of food borne illness resulting from consumption of fresh produce was the second highest out of all foodborne disease incidences. During this period fresh produce also had highest number of reported illnesses per outbreak compared to beef, poultry, seafood and eggs, (DeWaal and Bhuiya 2007). In the USA, between 1973 and 1987, fruit and vegetables were the cause of 2% of the foodborne disease outbreaks. However, by the 1990s, fresh produce had risen to be 6% of all reported foodborne outbreaks with over 16,000 cases of illness identified. Another study indicated that many as 13% of disease outbreaks in the USA may be attributed to fresh produce contaminated with pathogens (Hanning *et al.* 2009). The Center for Disease Prevention and Control (CDC) reported a substantial increase of outbreaks due to fresh produce in USA between 2006 and 2011 (Callejón *et al.* 2015) and the same trend was observed in the European Union where produce related outbreaks increased from 29 reports in 2006 to 44 in 2010 (Callejón *et al.* 2015).

There are many pathogens that are associated with foodborne disease outbreaks involving fresh produce. The following pathogens of concern have been reported as a source of contamination of fruit and vegetables when eaten raw: *Salmonella* spp., *Shigella* spp., *Escherichia coli*, *Campylobacter* spp., *Yersinia enterocolitica*, *Listeria monocytogenes*, *Staphylococcus aureus*, *Clostridium* spp., *Bacillus cereus*, *Vibrio* spp. and a number of other viruses and parasites (Islam *et al.* 2004a; Goodburn and Wallace 2013). Of these, bacterial

pathogens and their outbreaks are of the utmost concern in terms of illness severity and number of persons at risk of infection globally (Beuchat 1996). The bacterial pathogens, *Salmonella* spp. and *E. coli* O157:H7, are the major enteric pathogens reported to contribute to outbreaks of foodborne illness linked to fresh produce (Buck *et al.* 2003; Franz and van Bruggen 2008; Warriner *et al.* 2009; Olaimat and Holley 2012; Ongeng *et al.* 2015). These two bacterial pathogens are important because they cause disease outbreaks at low infection doses and are able to survive at typical home refrigerator temperatures (Ongeng *et al.* 2015). In addition, *Salmonella* is the second most prevalent causative agent (after norovirus) in produce-related outbreaks causing 18% and 20% of infections in the USA and the European Union, respectively (Callejón *et al.* 2015). The other important bacterial pathogen associated with fresh produce, especially ready-to-eat vegetables is *L. monocytogenes* (Little and Gillespie 2008; Sant'Ana *et al.* 2012a; Sant'Ana *et al.* 2012b).

2.2 Pathogens of concern: *Salmonella* spp. and *Listeria monocytogenes*

Salmonellae are Gram-negative, facultative anaerobic, non-spore forming and rod-shaped bacteria within the family Enterobacteriaceae. Apart from *S.* Pullorum and *S.* Gallinarum (which lack flagella), the members of this genus have peritrichous flagella which allows motility (Agbaje *et al.* 2011; Abakpa *et al.* 2015). They are non-lactose fermenting, urease-negative, oxidase-negative, acetylmethyl carbinol-negative, citrate-utilising and potassium cyanide-negative (Holt and Chaubal 1997; Abakpa *et al.* 2015). *Salmonella* and *L. monocytogenes* are both commonly encountered in animal manures and are known to survive for considerable periods in adverse environments (Himathongkham *et al*. 1999; Himathongkham and Riemann 1999).

2.2.1 *Salmonella* nomenclature

Theobald Smith, who was a pioneer epidemiologist, bacteriologist and pathologist, formally discovered *Salmonella enterica* in the 1880s. The genus *Salmonella* was named after Smith's chief supervisor and collaborator, Daniel E Salmon (Schultz 2008). The two species of *Salmonella* are *S. bongori* and *S. enterica*; the latter having over 2,500 serovars. *Salmonella* serovars identified after 1966 are designated by their antigenic formula, with each serovar being considered distinct strains within subspecies. However, frequently encountered serovars that were identified before 1966 are still often described by their original names rather than antigenic formula, which often indicated the syndrome, host specificity or

geographical region where it was first isolated (Brenner *et al.* 2000; Heyndrickx *et al.* 2005; Mestrovic 2015).

Over time, many scientists have tried to improve the taxonomy of *Salmonella* until the White-Kauffmann-Le Minor system was established to list all identified *Salmonella* serovars. This list is regularly updated by the World Health Organization (WHO) (Grimont and Weill 2007). The current nomenclature (Table 2.1) used by the CDC is based on recommendations from the WHO Collaborating Center. *Salmonella enterica* is divided into six subspecies (Popoff *et al.* 2001; Popoff *et al.* 2003; Ellermeier and Slauch 2006). Nearly all important human pathogenic isolates of *Salmonella* are from *S. enterica* subsp. *enterica*, and strains in this subspecies cause the majority of *Salmonella* illness in humans. Serotypes in *S. enterica* subspecies II (*S. enterica* subsp. *salamae*), IIIa (*S. enterica* subsp. *arizonae*), IIIb (*S. enterica* subsp. *diarizonae*), IV (S. *enterica* subsp. *houtenae*), V (*S. bongori*), and VI (*S. enterica* subsp. *indica*) are rarely isolated from humans but are prevalent in the environment (Brenner *et al.* 2000; Ellermeier and Slauch 2006). In comparison, *S. enterica* subsp. *enterica* is mostly isolated from warm-blooded animals. When describing *Salmonella* serovars, the species name is often dropped; for example, *S. enterica* subsp. *enterica* serovar Enteritidis may be referred to as *S.* Enteritidis (Table 2.2). There are more than 1450 serovars within *S. enterica* subsp. *enterica* (Popoff *et al.* 2004; Klerks 2007; Agbaje *et al.* 2011; CDC 2015).

Table 2.1 *Salmonella* species, subspecies and serovars according to the White-Kauffmann-Le Minor naming system. Adapted from Brenner *et al*. (2000).

***Salmonella* species and subspecies**	**No. of serovars within subspecies**
S. enterica subsp. *enterica* (I)	1,454
S. enterica subsp. *salamae* (II)	489
S. enterica subsp. *arizonae* (IIIa)	94
S. enterica subsp. *diarizonae* (IIIb)	324
S. enterica subsp. *houtenae* (IV)	70
S. bongori (V)	20
S. enterica subsp. *indica* (VI)	12
Total	**2,463**

Table 2.2 *Salmonella* nomenclature in use at the Center for Disease Control and Prevention (CDC 2000). Adapted from Brenner *et al.* (2000).

Taxonomic position	Nomenclature
Genus (italics)	*Salmonella*
Species (italics)	*enterica*, which includes subspecies I, II, IIIa, IIIb, IV and VI
	bongori (formerly subspecies V)
Serotype (capitalised, not italicised to avoid confusion between serovars and species)	The first time a serovar is mentioned in the text, the name should be preceded by the word "serotype" or "ser"
	Serovars are named in subspecies I and designated by antigenic formulae in subspecies II to IV, and VI and *S. bongori*
	Members of subspecies II, IV, and VI and *S. bongori* retain their names if named before 1966

2.2.2 *Salmonella* identification and serotyping

2.2.2.1 The White-Kauffmann-Le Minor system

There are more than 2500 serovars in the genus *Salmonella* based on their antigenic formula. According to the White-Kauffmann-Le Minor system, serological groups of *Salmonella* are based on the composition of O-antigens associated with the bacteria surface and the H-antigens on the threadlike flagella. To date, 57 O-antigens and 117 H-antigens have been identified. (Iankov *et al.* 2002; Fitzgerald *et al.* 2003).

The structure of the O-antigen is very variable among strains of *Salmonella* and subtle chemical changes alter O-antigen structure which can have profound effects on antibody recognition (Kim and Slauch 1999). Generally, the O-antigen is used to assign the *Salmonella* to a group, while the H-antigen may be used to confirm the serovars within the group. For instance, *Salmonella* serovars Sofia and Typhimurium belong to the same O-antigen grouping (group B) but they have different H-antigenic formulae (see Chapter 3, Table 3.1). The H-antigen, sometimes referred to as the O-group, can exist as two serological formats known as phase 1 and phase 2. A third antigen, known as the Vi-antigen, may occur in other *Salmonella* serovars. This Vi-antigen has the potential to interfere with the activity of O-antigens and therefore has to be deactivated to allow accurate serological identification (Cai *et al.* 2005; Sonne-Hansen and Jenabian 2005; Ellermeier and Slauch 2006).

To identify the *Salmonella* serovar the microorganism in question is exposed to the antiserum that contains the characteristic *Salmonella* antibody. Positive identification occurs when the *Salmonella* bacteria physically clump together with the homologous antiserum (Cai *et al.* 2005; Wattiau *et al.* 2011). The agglutination reaction or clumping of the antigen-antibody can easily be observed within a minute of mixing. A positive result is obtained when one or more of the antigens interact with the antiserum. No agglutination indicates a negative result and suggests the serovar is from another grouping.

Serotyping is usually initiated by testing the isolate with polyvalent O-antiserum. The majority (about 98%) of *Salmonella* encountered in warm-blooded animals possess an O-antigen corresponding to the agglutinins contained in OMA, OMB and OMC sera (Figure 2.1). The OMA, OMB, OMC and OMD are a pool of groups of sera that are also known as polyvalent sera. Polyvalent 'O' (somatic) antisera are intended to aid initial serogrouping. For example, if a strain tests negatively with OMA (pool sera), then positively with OMB, there is no need to check for the other sera, rather, further checking with OMB is required to characterise within the OMB group (Hendriksen *et al.* 2009; Wattiau *et al.* 2011; CDC 2015). In the OMB group, there are a number of sera such as: (i) O:11, (ii) O:6,7,8, (iii) O:13,22,23, and (iv) O:6,14,24 which are known as monovalent sera and full identification of the O-antigens can be achieved using monovalent specific O-antisera. Therefore, if there is no agglutination for O:11, the next test will be using O:6,7,8, and if agglutination is obtained, checking for the rest of the sera group is not required (Cai *et al.* 2005; Hendriksen *et al.* 2009; Wattiau *et al.* 2011; Cox and Pavic 2014). Based on the O-antigen, Salmonellae are categorised in group A, B, C1, D, E1, E4 and so on.

Individual serotypes in each O-group are characterised using polyvalent and monovalent H-antisera. Each O- and H-antigen has a unique code (CDC 2015). As the antigenic formula with O, H-phase 1 and H-phase 2 are identified, the serotyping is confirmed by referring to a reference catalogue such as the White-Kauffmann-Le Minor scheme (Anonymous 2013). For example, *S.* Enteritidis belongs to group D (O:9), and is characterised by its O (9,12) and H (g, m) antigens. An individual *Salmonella* cell expresses either phase I or phase 2 H-antigens, but not both. Therefore, the format for writing a serovar is: Subspecies [space] O-antigens [colon] H-phase 1 antigen [colon] H-phase 2 antigen. *S.* Typhimurium, for example, belongs to subspecies I, group B (O4), has four O antigens (1,4,[5],12), and one H-phase 1 flagellar

antigen (i) and two H-phase 2 antigens (1,2). The antigenic formula is 1,4,[5],12:i:1,2 (Cai *et al.* 2005; Grimont and Weill 2007).

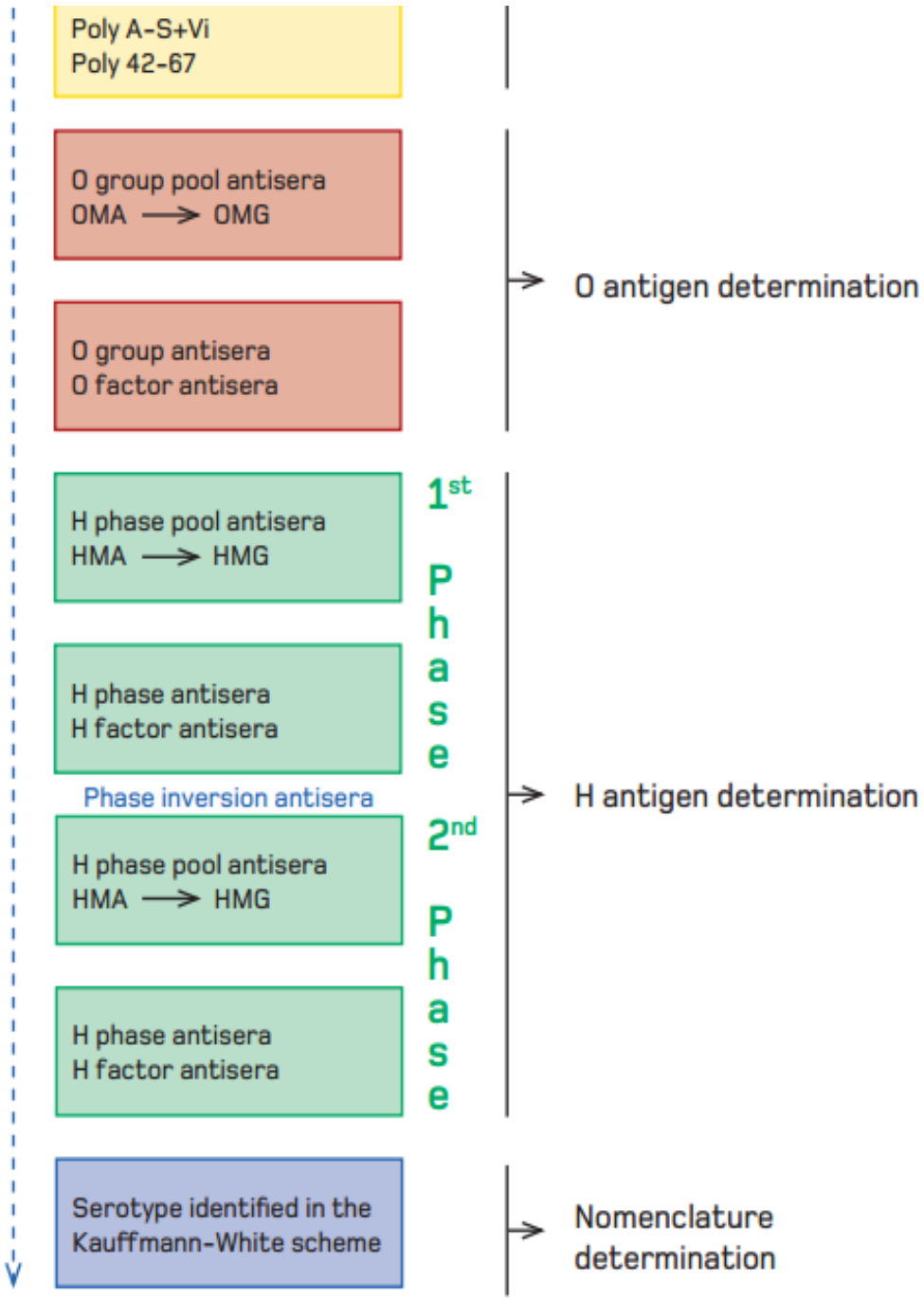


Figure 2.1 Flowchart showing *Salmonella* serotyping. From Anonymous (2013).

2.2.2.2 Molecular methods

Molecular identification of serovars can be done by restriction endonuclease digestion, nucleic acid amplification, or nucleotide sequencing techniques (Imen *et al.* 2011). Up until the advent of whole genome sequencing, Pulsed Field Gel Electrophoresis (PFGE) was the most widely accepted method of identification. The DNA is first cut with restriction enzymes and the fragments separated by electrophoresis using multi directional pulsed currents. Identification is reliant on characteristic separation patterns of the DNA fragments which can be up to 1200 kb (Imen *et al.* 2011). However, this method takes time, is labour-intensive and can produce false positives when compared with other molecular methods (Wattiau *et al.* 2011).

Amplified Fragment Length Polymorphism (AFLP) amplifies adapter-specific restriction fragments by Polymerase Chain Reaction (PCR) with specifically designed primers (Imen *et al.* (2011). The disadvantage with this method is the need for considerable technical expertise.

In addition to these two molecular methods, plasmid proofing, ribotyping, insertion sequence (IS) typing, randomly amplified polymorphic DNA (RAPA), multilocus sequence typing (MLST), and multiplex PCR are also used for *Salmonella* serotyping.

2.2.3 *Salmonella* serovars used in the present study

All the serovars used in the present study belong to *S. enterica* subsp. *enterica*. Because of their role in foodborne diseases associated with the poultry and fresh produce industries, *Salmonella* serovars Enteritidis, Infantis, Montevideo, Sofia, Typhimurium and Zanzibar were used as test serovars. The increased incidence of *S.* Enteritidis in poultry since the 1970s has made these serovar a particularly prevalent source of food poisoning related primarily to poultry products but also including fresh produce (Greig and Ravel 2009; OzFoodNet Working Group 2009; Food Satey News 2013).

From 2008 to 2009, *S. enterica* serovars Typhimurium, Montevideo and Infantis were among the top five listed *Salmonella* infections in all Australian states except Western Australia (OzFoodNet Working Group 2010). *S.* Enteritidis is the most common strain of *Salmonella* in the food industry and it is the most frequent cause of human illness internationally. In the last 20 years, *S.* Enteritidis has become the single most common cause of food poisoning in

the USA. According to the CDC, 95% of *Salmonella* infections come from foodborne sources and serovars Typhimurium and Enteritidis are associated with over half of the reported infections. In addition, these two serovars have a wide host range since they cause disease in many animals (Fatica and Schneider 2011). *S.* Typhimurium, which is almost as common and *S.* Enteritidis, exhibits antibiotic-resistance making it difficult to eliminate in animal production systems and when treating affected humans (Food Satey News 2013). In Australia, *S.* Typhimurium is more common than *S.* Enteritidis. In 2008 more than 40% of all notified outbreaks was attributed to *S.* Typhimurium making it the most common serovar (Greig and Ravel 2009; OzFoodNet Working Group 2009). In another report, *S.* Typhimurium accounted for 95% of the *Salmonella* notifications in Australia in 2014, and from the total of 709 notifications, there were 64 and 18 reports for serovars Enteritidis and Infantis, respectively (OzFoodNet 2014). *S.* Sofia is an avirulent form of *Salmonella* and is the most prominent serovar in Australia. It is only found in Australia, and mainly isolated from poultry (Mellor *et al.* 2010). Another serovar, Zanzibar, was reported as a cause of foodborne disease outbreaks in New South Wales Australia in 2013 (OzFoodNet 2013).

2.2.4 Salmonellosis

Ingesting *Salmonella* bacteria may cause Salmonellosis. This disease can be manifested in two forms; namely typhoid fever or non-typhoidal gastroenteritis. *Salmonella* normally resides in animals and is transmitted to humans either directly or indirectly through soil, water or food (Klerks 2007). The dose of *Salmonella* cells required to cause infection was thought to be high, however for *S.* Typhimurium, very low amounts (10–100 cells) have been reported to cause a number of outbreaks (Jyoti *et al.* 2016). The dose required to cause infection depends on the food type, the serovar, the physiological form of the bacteria and the susceptibility of the host (Darwin and Miller 1999). Salmonellosis has an important economic and social impact because of its prevalence in most countries, the occurrence of *Salmonella* in farm animals and its transmission to humans (Agbaje *et al.* 2011).

2.2.5 *Salmonella* detection and isolation

Methods for detecting and isolating *Salmonella* spp. involve pre-enrichment of the sample in nonselective media, enrichment in selective media and plating onto selective/differential agar. Individual colonies are then subjected to biochemical/serological confirmations. The culture methods used in this study were modified from the Food and Drug Administration (FDA)

standards as set out in the Bacteriological Analytical Manual (Andrews *et al.* 2013) and follow Harris *et al.* (2012).

2.2.5.1 Pre-enrichment

Due to various injuries caused by heating, freezing and drying, *Salmonella* in food or environmental samples has been reduced over time (Wang and Hammack 2014). Bacterial numbers may also decrease during storage and transportation. The injured *Salmonella* cells are enriched in a pre-enrichment solution which allows the cells to revive and proliferate (Hoorfar and Baggesen 1998; Wang and Hammack 2014). A sequential enrichment in nonselective and selective media allows for enhanced detection and recovery of sub-lethally injured *Salmonella*.

Pre-enrichment media are nonselective and generally contain nutrients required for cell growth and multiplication, repair of cell injury, rehydration, and dilution of toxic or inhibitory substances. Some pre-enrichment media include nutrient supplements to support the resuscitation of injured cells (Wang and Hammack 2014). Buffered peptone water (BPW) is one of the most widely used pre-enrichment broths for *Salmonella*. It is used to buffer the pH of the growth medium and support metabolism of microorganisms during the enrichment process in addition to providing conditions for the resuscitation and growth of cells prior to selective enrichment (Baylis *et al.* 2000; Wang and Hammack 2014). Pre-enrichment broths for *Salmonella* are generally incubated at 37°C for 18–24 h. After pre-enrichment, a portion of the pre-enrichment broth is sub-cultured on selective enrichment media.

2.2.5.2 Selective enrichment

Selective enrichment media contain compounds that suppress the growth of competitive microorganisms while allowing *Salmonella* to proliferate. Selective media are routinely used in the recovery of *Salmonella* from a wide variety of environmental and food samples (Wang and Hammack 2014).

Tetrathionate broth base (TTB) is recommended by the FDA as a selective enrichment broth to isolate and detect *Salmonella* spp. When iodine-potassium iodide (I_2-KI) solution is added in the medium, tetrathionate is formed and this serves a selective agent, combined with sodium thiosulfate, to suppress coliforms and other microflora. Calcium carbonate is used to neutralise toxic metabolites during the incubation process. The amount of pre-enrichment

broth sub-cultured using selective media should be sufficiently small (e.g. 1:9) so that it does not interfere with selectivity (Wang and Hammack 2014).

The second selective enrichment containing nutrients necessary for favourable *Salmonella* growth and flagella development is mBroth (Forsythe and Hayes 1998). Another broth recommended by the FDA for enrichment of *Salmonella* is Rappaport-Vassiliadis (RV). This media is a selective enrichment medium that is used after pre-enrichment broths. This particular medium was first developed by Rappaport *et al*. (1956) and modified by Vassiliadis *et al*. (1976; as cited in Vassiliadis (1983)) to selectively enrich *Salmonella* spp. but not other species from other Enterobacteriaceae. Rappaport-Vassiliadis contains tryptone to provides amino acids and other nitrogenous substances, potassium dihydrogen phosphate as a buffer, magnesium chloride hexahydrate to raise the osmotic pressure of the medium and malachite green oxalate to inhibit the growth of microorganisms other than *Salmonella* spp. (Wang and Hammack 2014).

2.2.5.3 Selective agar for enumeration and enrichment

To isolate and differentiate *Salmonella* from other microorganisms either from extracted soil samples or from incubated selective enrichment media, samples are plated on selective agar media. Selective plating media suppress the growth of some competitive microflora while allowing the growth of distinct and well-isolated colonies of *Salmonella*. Selective media usually contain the necessary nutrients for the growth and fermentation of the specific bacteria and indicator dyes to show the production of hydrogen sulfide (H_2S) and changes in pH. They also contain one or more inorganic salts to maintain the osmotic balance in the medium (Wang and Hammack 2014).

Xylose lysine deoxycholate (XLD) agar is used to distinguish *Salmonella* from competitive microflora such as *E. coli*. It contains sodium deoxycholate as a selective agent to inhibit Gram-positive organisms. Production of H_2S is mainly detected by the presence of sodium thiosulfate and ferric ammonium citrate and any changes from fermentation and decarboxylation reactions are detected in the media by phenol red pH indicator (Wang and Hammack 2014). Differentiation of *Salmonella* spp. from nonpathogenic bacteria therefore relies on xylose fermentation, lysine decarboxylation and production of H_2S. Typical colonies on XLD agar appear pink with black centres and many *Salmonella* cultures are observed as colonies with large, shiny black centres or are wholly black (Wang and Hammack 2014; Sagar 2015). The other agar media that is used for detection and isolation of non-Typhi

Salmonella based on its selectivity and colony characterization is Xylose Lysine Tergitol 4 (XLT4). It uses the surfactant Tergitol™ 4 to inhibit growth of non-*Salmonella* species. Therefore can be used to differentiate other organisms using the same mode of action as XLD agar. Compared to XLD agar, XLT4 markedly inhibits the growth of *Enterobacter aerogenes, E. coli, Proteus, Pseudomonas, Providencia, Alteromonas putrefaciens, Yersinia enterocolitica* and *Acinetobacter calcoaceticus*. Typical colonies on XLT4 agar are black or are yellow with a black centre after 18–24 h of incubation. After extended incubation, *Salmonella* colonies on XLT4 agar turn totally black or are pinkish red with black centres. H_2S-negative colonies of *Salmonella* are pink to yellow (Wang and Hammack 2014).

Chromogenic agars differentiate bacterial species on the basis of colour using selective and differential agents in the media. Inclusion of X-gal and magenta-caprylate allows for identification of *Salmonella* spp (Maddocks *et al.* 2002; Cassar and Cuschieri 2003; Wang and Hammack 2014). These chromogenic substrates, together with specified selectivity of the medium, are the principle behind chromogenic media. Specific enzymes in the targeted organisms are responsible for the production of chromophores which are produced with cleavage of chromogen. A distinct colour change is observed in the medium upon the release of chromophores (e.g. esterase activity in *Salmonella*) (Perry and Freydiere 2007). Despite the reliability and high sensitivity and specificity of *Salmonella* chromogenic media, other media such as XLD should also be used to evaluate samples for the presence of *Shigella* spp. (Maddocks *et al.* 2002).

2.2.6 *Listeria monocytogenes*

The second bacterial pathogen investigated in this study, albeit less thoroughly, is *L. monocytogenes*. This bacteria was first described by Everitt Murray and his co-workers in 1926 (Bisha 2009) after isolating a previously undescribed bacterium from the blood of diseased and dead rabbits. The genus *Listeria* includes several species: *L. ivanovii, L. seeligeri, L. innocua, L. welshimeri and L. monocytogenes. Listeria monocytogenes* is the most common species causing disease in humans and animals although there have been sporadic reports of *L. innocua* and *L. seeligeri* causing disease in humans (Batt 2014). *Listeria monocytogenes* is thought to consist of four lineages with most isolates and the majority of human pathogenic forms belonging to lineages I and II (Orsi *et al*. 2011).

Listeriae are small Gram-positive rods, but they can sometimes appear as cocci. They are non-spore-forming bacteria and they do not form capsules. They are urease-negative, catalase-positive and oxidase negative and are able to hydrolyse aesculin and exhibit tumbling motility at room temperature (Bisha 2009). The temperature range for growth is between <1°C to approximately 50°C, with an optimum temperature of 30–37°C. *Listeria monocytogenes* is quite hardy and is frequently found in the environment. The organism can withstand freezing, but it is inactivated by heating at 60°C for 30 minutes (Batt 2014). *Listeria monocytogenes* is only considered to be a serious disease of immuno-compromised individuals and pregnant women, neonates (Harris *et al.* 2003). Recent research suggests that infectious doses may be higher than previously thought such that infectious does for two strains of *Listeria* (12443 and Scott A) ranged from 7.2 x 10^6 CFU for strain 12443 to 3.7 x 10^{10} CFU for Scott A strain in pregnant monkeys (Smith *et al.* 2003).

2.3 Incidence of pathogenic *Salmonella* and *Listeria* on fresh produce

As the global dietary intake of fruit and vegetables has risen, so too have the reports of foodborne illness outbreaks linked with consuming fresh produce, due to *Salmonella*, *E. coli* O157:H7 and *L. monocytogenes* (Warriner *et al.* 2009; Park *et al.* 2012). These bacterial pathogens are among the most concerning in regards to produce safety (Beuchat 2002; Franz and van Bruggen 2008). The present study focuses on *Salmonella* as it is more often found to be the most common pathogenic agent and there are more incidences of disease related to fresh produce outbreaks than *E. coli* O157:H7 ((Sivapalasingam et al. 2004; Klerks 2007). The second pathogen of concern in this study is *L. monocytogenes*, which is found frequently in the environment.

Every year in the USA, *Salmonella* is throught to cause approximately 1 million foodborne illnesses with 19,000 hospitalisations and 380 deaths (CDC 2016). In a review of outbreaks of foodborne illness associated with single items of fresh produce from 1990–2004 (Brandl 2006), *E. coli* O157:H7 and *S. enterica* were found to be the most frequent bacteria in these outbreaks. The latter group caused 30% of outbreaks linked to fruit and leafy vegetables, whereas, *E. coli* O157:H7 caused 48% of infections (Olaimat and Holley 2012). Evaluation of 1,183 fresh fruit and vegetable samples taken from produce grown in Ontario during the summer of 2004, showed that Roma tomatoes and organic lettuce tested positive for *Salmonella* (Arthur *et al.* 2007).

In 23 European Union countries in 2005, more than 5,300 foodborne disease outbreaks were reported, of which 64% were due to *Salmonella* (Franz and van Bruggen 2008). In another survey, 83 produce-associated outbreaks (5.5% of the total number) were reported in England and Wales between 1992 and 2000, with *Salmonella* having the highest incidence of reporting (Long *et al.* 2002). In 2002, two notable outbreaks that involved 314 cases of *Salmonella* infections in the United Kingdom were linked to the consumption of lettuce (Islam *et al.* 2004a). More than 50,000 people suffered from Salmonellosis between 1999 and 2000 in the Netherlands (Bouwknegt *et al.* 2003) and in 2002, the incidence of Salmonellosis was approximately 35,000 cases in the general population (Bouwknegt *et al.* 2004). In the Netherlands, an estimated total cost of €33–91 million is spent annually for Salmonellosis (Wannet *et al.* 2003). In a Spanish survey in 2005–2006, high microbial loads were associated with grated carrot, arugula and spinach: 6.2, 5.3 and 6.0 log CFU g^{-1} of Enterobacteriaceae compared to 7.8, 7.5 and 7.4 log CFU g^{-1} of aerobic mesophilic microorganisms; 6.1, 5.8 and 5.2 log CFU g^{-1} of yeast and moulds; and 5.9, 4.0 and 5.1 log CFU g^{-1} lactic acid bacteria (Abadias *et al.* 2008).

In Australia and after Campylobacteriosis, Salmonellosis is the most frequently reported cause of gastric sickness (Yates 2011). In 2001, 41 cases of *Salmonella* infection were associated with contaminated lettuce (Stafford *et al*. 2002). The Salmonellosis notification rate varies among localities with a low of 31 cases per 100,000 people reported in Victoria to a high of 226 cases per 100,000 people in the Northern Territory. Young children under four years usually are the most susceptible, with 300 cases per 100,000 population reported in 2008 (OzFoodNet Working Group 2009).

Outbreaks due to *L. monocytogenes* are mostly associated with ready-to-eat vegetables. In 2011, an outbreak due to the consumption of *Listeria*-contaminated cantaloupe that was distributed to 28 states in the USA resulted in 146 illnesses, 30 deaths and one miscarriage (CDC 2011e). *Listeria monocytogenes* is commonly found on raw fruit and vegetables and plant material (Cordano and Jacquet 2009) and grows at refrigeration temperatures which increases the risk to consumers of ready-to-eat foods (Jay-Russell 2013).

For the period from 2005–2011, *Salmonella* and *E. coli* contributed 49 and 47%, respectively, of the total cases of illness (9,520) associated with fresh produce in different parts of the world (Olaimat and Holley 2012). More than half of the outbreaks reported during this period were from the USA. The largest outbreak in terms of human illness occurred in Europe in

2011 due to *E. coli* O104:H4-contaminated vegetable sprouts which led to 3,911 foodborne illnesses and 47 deaths (Table 2.3). In Australia in 2006, *Salmonella* linked with alfalfa sprouts and canteloupe, caused 125 and 115 cases, respectively (Table 2.3). Lettuce, spinach and alfalfa/mixed sprouts contributed to more than 50% of the foodborne illness outbreaks. A *Salmonella* outbreak in 2013 in the USA caused by contaminated cucumber resulted in 28% of ill persons being hospitalised but no deaths were reported.

2.4 How produce gets contaminated: sources of contamination

Food safety encompasses food contaminated by physical, chemical or biological hazards. Of the three, microbial hazards tend to generate a more negative effect amongst the public. This might be due the regular reporting of biological hazards, the fact they tend to affect a large number of consumers and because they generally induce instant and severe symptoms (Franz and van Bruggen 2008).

Any disease causing-agent that comes in contact with fresh produce, from the production line to the processing chain, has the potential to be a contaminant source. Examples of contamination sources include irrigation water, manure, compost, animals and soil (Abadias *et al.* 2006). Many studies have reported that animal manure and irrigation water represent the two most important sources of pathogen transmission from animal hosts to fresh produce in the preharvest stage (Semenov 2008; Park *et al.* 2012). Moreover, Franz and Van Bruggen (2008) reported that the application of manure or compost is the most important consideration for contamination of vegetables in the field. Most produce-associated disease outbreaks in Europe were linked to whole products exposed to soil and/or water during production while processed fruit and vegetables had fewer outbreaks (Anonymous 2002).

Table 2.3 Foodborne illness outbreaks linked to fresh produce from 2005–2011. Adapted from Olaimat and Holley (2012) and CDC (2017). USA = United States of America; UK = United Kingdom.

Location	Year	Pathogen	Produce	Cases (deaths)
Canada	2005	*Salmonella*	Mungbean sprouts	592
USA	2005	*Salmonella*	Tomato	459
USA	2006	*E. coli* O157:H7	Spinach	199 (3)
Australia	2006	*Salmonella*	Alfalfa sprouts	125
USA, Canada	2006	*Salmonella*	Fruit salad	41
USA	2006	*Salmonella*	Tomato	183
USA	2006	*E. coli* O157:H7	Lettuce	81
Australia	2006	*Salmonella*	Cantaloupe	115
USA	2006	*E. coli* O157:H7	Spinach	22
Europe	2007	*Salmonella*	Baby spinach	354
USA, Europe	2007	*Salmonella*	Basil	51
Australia, Europe	2007	*Shigella sonnei*	Baby carrot	230
Europe	2007	*Salmonella*	Alfalfa sprouts	45
USA, Canada	2008	*Salmonella*	Pepper	1,442 (2)
USA, Canada	2008	*E. coli* O157:H7	Lettuce	134
UK	2008	*Salmonella*	Basil	32
USA	2008	*Salmonella*	Cantaloupe	51
USA	2009	*Salmonella*	Alfalfa sprouts	235
USA	2010	*E. coli* O145	Lettuce	26
USA	2010	*Salmonella*	Alfalfa sprouts	44
USA	2010	*L. monocytogenes*	Fresh cut produce (celery)	10 (5)
USA	2011	*Salmonella*	Alfalfa and mixed sprouts	140
USA	2011	*Salmonella*	Cantaloupe	20
USA	2011	*Salmonella*	Papaya	106
Europe	2011	*E. coli* O104:H4	Vegetable sprouts	3,911 (47)
USA	2011	*L. monocytogenes*	Cantaloupe	146 (31)
USA	2011	*E. coli* O157:H7	Strawberry	15 (1)
USA	2011	*E. coli* O157:H7	Lettuce	60
USA	2011	*L. monocytogenes*	Cantaloupe	146 (30)
USA	2013	*Salmonella*	Cucumber	84
USA	2014	*Salmonella*	Cucumber	275
USA	2015	*Salmonella*	Bean sprouts	115
USA	2016	*Listeria*	Packaged salad	19 (1)

Application of animal manure is encouraged as a sustainable means of adding fertiliser in salad production systems and to improve overall soil helath and biodiversity. It is considered to be an important component of a globally sustainable agricultural industry (Holley *et al.*

2006; Franz and van Bruggen 2008). The use of manure in organic farming is expected to increase with the acceptance of organic produce (Islam *et al.* 2004a) yet, if any manure contains human pathogenic bacteria, it can cause contamination of fresh produce when applied to fields (Franz and van Bruggen 2008). The level of pathogens varies based on the different manure types and the storage time (Table 2.4). In particular, untreated or partially composted manure may contain large numbers of pathogenic organisms, especially enteropathogenic bacteria (Semenov 2008).

It has been reported that poultry are a known vector for *Salmonella* and while cattle and sheep manure can be a primary sources of *Salmonella* and *E. coli* O157:H7 (Olaimat and Holley 2012). As a result, the practice of adding manure in leafy vegetable production has been questioned with respect to food safety, especially in organic vegetable production, since this substrate introduces enteric pathogens into the food chain.

Contaminated soil can be a reservoir of enteric pathogens with these pathogens cycling through the environment and into the food system (Figure 2.2). The transmission of pathogens from manure through the production chain has become more frequent (Natvig *et al.* 2002) and is one of the major causes for the increased outbreaks of diseases from the consumption of fruit and vegetables (Beuchat 1996; Sivapalasingam *et al.* 2004). Enteric pathogens may transfer to crops through the application of contaminated, fresh or improperly composted manure. Soil can be contaminated not only from compost/manure but also directly or indirectly by humans, animals, water, and waste (Figure 2.2).

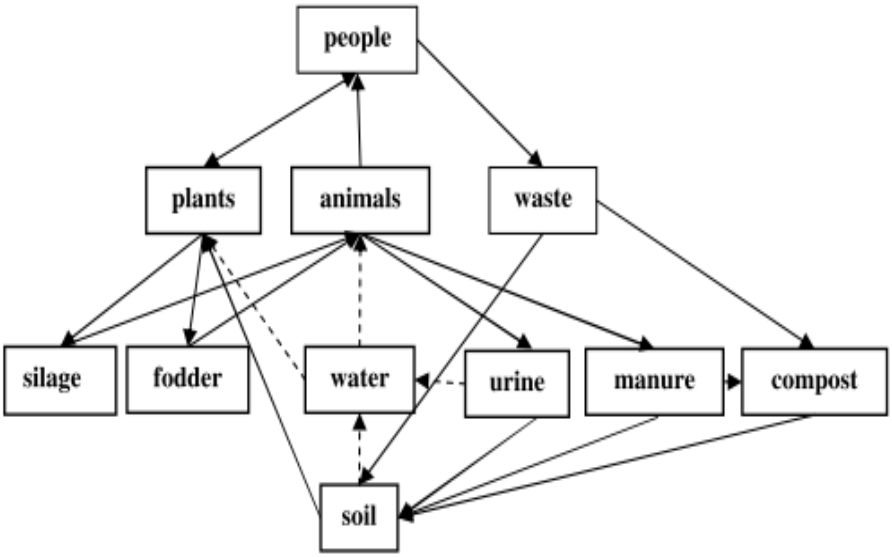


Figure 2.2 Microbial cycle of enteropathogens. From Semenov (2008).

Table 2.4 Summary of the levels of zoonotic pathogens observed in livestock manures containing zoonotic agents. Data shown are arithmetic (A) mean for positive isolations only and highest levels observed for each pathogen and manure type are also shown (M). The number of positive isolations used to calculate each mean is shown (n). Adapted from Hutchison *et al*. (2004a). ND = not determined.

	Amount of pathogens (CFU $\log_{10}$ g^{-1}) found in positive livestock waste types							
	Cattle		**Pig**		**Poultry**		**Sheep**	
Zoonotic agent	**Fresh**	**Stored**	**Fresh**	**Stored**	**Fresh**	**Stored**	**Fresh**	**Stored**
E. coli O157								
A	2.9×10^6	8.6×10^3	6.9×10^4	4.5×10^3			1.1×10^4	2.5×10^3
M	2.6×10^8	7.5×10^4	7.5×10^5	1.8×10^4	ND	ND	4.9×10^4	5.0×10^3
n	107	39	15	9			5	2
Salmonella								
A	3.9×10^4	1.9×10^5	9.6×10^3	8.9×10^2	5.0×10^3	4.7×10^3	1.1×10^3	5.8×10^3
M	5.8×10^5	7.2×10^6	7.8×10^4	2.0×10^3	2.2×10^4	8.0×10^3	2.0×10^3	5.8×10^3
n	62	43	10	3	12	3	2	1
Listeria								
A	1.5×10^4	2.2×10^4	4.6×10^4	1.6×10^4	3.2×10^4	5.6×10^2	4.5×10^2	2.1×10^3
M	4.2×10^5	9.8×10^5	9.7×10^5	1.5×10^5	1.9×10^5	1.3×10^3	1.7×10^3	8.1×10^3
n	241	133	25	11	13	4	7	4

2.5 Survival of bacterial pathogens and factors affecting survival

Long term survival of *Salmonella* and other enteric pathogens in water, manure or compost, soil and sediment has been well documented in many studies (Cools *et al.* 2001; Gong *et al.* 2005; Eamens 2006; Danyluk *et al.* 2008; Ongeng *et al.* 2015). An overview of the survival time in different matrices, specifically for *Salmonella*, under different experimental settings is shown in Table 2.5.

In a survival study of *E. coli* O157:H7 and *S.* Typhimurium in sandy soil, *E. coli* O157:H7 numbers declined faster compared to *Salmonella* (Franz *et al.* 2005). *S.* Typhimurium is highly resistant to a range of environmental stresses and adapts readily in the nevironment to enable survival for longer periods of time than *E. coli* O157:H7 (Semenov *et al.* 2007). In a comparative study, *E. coli* O15:H7 strain B6-914 GFP-91 (where GFP is green fluorescent protein) was reported to survive for 154–271 days (Islam *et al.* 2004a) and for 154–196 days (Islam *et al.* 2005) in cattle manure-amended soil in the field. In a similar study, *E. coli* O157:H7 (strain ACCC 43888) and *S.* Typhimurium were found to survive (below the limit of detection, 2 CFU g^{-1}) from 4–12 and 4–6 weeks, respectively, in cattle manure and soils amended with cattle manure in greenhouse and field conditions (Ongeng *et al.* 2011). In another field setting, *S.* Typhimurium was reported to survive (not detected by direct plating) for 203–231 days in bovine manure-amended soil (Islam *et al.* 2004c) and for 161–231 days in compost-amended soil (Islam *et al.* 2004a).

A multidrug-resistant and a drug-susceptible serovar of *S.* Newport survived up to 405 days at 25°C in bovine manure-amended soil. In another study, a cocktail of five *Salmonella* serovars in hog manure slurry was reported to survive for more than 300 days at 4°C (Table 2.5). The survival times in Table 2.5 reflect variation in survival in different environments and experimental methods (Ongeng *et al.* 2015).

Factors such as temperature, pH, salt concentration, soil moisture, soil type, nutrient availabilty, microbial species and diversity all influence the survival of human pathogenic bacteria introduced into the soil habitat (van Veen *et al.* 1997; Franz and van Bruggen 2008; Fornefeld *et al.* 2017). This in turn affects the possible contamination of vegetables in the field (Semenov 2008).

Table 2.5 Overview of studies on survival of *Salmonella enterica* under different experimental conditions. Survival time is taken to be when counts are below the limit of detection. Adapted from Ongeng *et al.* (2015).

Strain used	Matrix used	Experimental conditions	Survival time (days)	Reference
S. Typhimurium DT104 (S811/99)	Bovine manure	Field setting	34–120	(Hutchison *et al.* 2004b)
S. Typhimurium DT104 (S10570/99)	Pig manure	Field setting	56–120	(Hutchison *et al.* 2004b)
S. Enteritidis PT4 (S8167/99)	Poultry manure	Field setting	56	(Hutchison *et al.* 2004b)
S. Typhimurium x^{3985} Δcrp- 11 Δ cya-12	Bovine manure	Field setting	203–231	(Islam *et al.* 2004c)
S. Typhimurium x^{3985} Δcrp- 11 Δ cya-12	Compost-amended soil	Field setting	161–231	(Islam *et al.* 2004b)
S. Typhimurium unspecified strain	Bovine manure	Field setting	32–42	(Hutchison *et al.* 2005)
S. Typhimurium unspecified strain	Pig manure	Field setting	16–32	(Hutchison *et al.* 2005)
S. Typhimurium unspecified strain	Sheep manure	Field setting	16	(Hutchison *et al.* 2005)
S. Typhimurium unspecified strain	Poultry manure	Field setting	63	(Hutchison *et al.* 2005)
S. Typhimurium MAE119–pGFP, Typhimurium MAE110–pGFP	Bovine manure	Isothermal at 20°C	>133	(Franz *et al.* 2005)
S. Newport unspecified strains	Cow manure	Isothermal at 25°C	184	(You *et al.* 2006)
S. Newport unspecified strains	Bovine manure-amended soil	Isothermal at 25°C	405	(You *et al.* 2006)
S. Newport unspecified strains	Bovine manure slurry	Storage in steel tanks	90	(Nicholson *et al.* 2005)
S. Newport unspecified strains	Bovine manure-amended soil	Field setting	300	(Nicholson *et al.* 2005)
Mixture of *S.* Typhimurium, *S.* Agona, *S.* Hadar, *S.* Oranienburg, all unspecified strains	Hog manure slurry	Isothermal at 4, 25 and 37°C	>300 at 4°C	(Arrus *et al.* 2006)
S. Typhimurium LT2, virulence attenuated	Bovine manure and manure-amended soil	Screen house and field settings	42–98	(Ongeng *et al.* 2011)

2.5.1 Soil moisture

Many studies have indicated that the moisture status of soil is an important factor affecting the survival of enteric pathogens (Entry *et al.* 2000; Mubiru *et al.* 2000; Holley *et al.* 2006; Lang and Smith 2007) such that low moisture availability in soil adversely affects survival of enteric bacterial pathogens in soil or manure-amended soil (Jamieson *et al.* 2002). In a survival study in a soil system held at 25°C, both *E. coli* O157:H7 and *Salmonella* was affected by moisture content with greater losses in soils adjusted to 40% water holding capacity (WHC) compared to soils adjusted to 20% WHC (Erickson *et al.* 2014). It was also reported that drying reduces the number of *S.* Typhimurium found in manure and litter, however, drying is only effective at certain levels of water activity. When most of the water has been removed, *Salmonella* will survive for longer periods of time (Himathongkham and Riemann 1999). A study modelling growth and death kinetics of *Salmonella* as a function of water activity (A_w) and pH reported that *Salmonella* population decreased approximately 5 log CFU g^{-1} in 9 hours to below detection limits, in chicken litter with a pH of 4 and a_w 0.84 (Payne *et al*. 2007).

2.5.2 Temperature

Salmonella can grow in a wide range of temperature (2–54°C) although in bacteriological media, growth below 7°C has been observed, while growth above 48°C is mainly for mutant and tempered strains (Cox and Pavic 2014). The optimum temperature for *Salmonella* growth is 37°C (Cox and Pavic 2014).

Generally, temperature has an important effect on the growth and decay of bacteria in the soil (Ongeng *et al.* 2015). Various studies have indicated that a decline in *Salmonella* numbers occurs with increasing temperature (Wang *et al.* 1996; Kudva *et al.* 1998; Himathongkham *et al.* 1999; Arrus *et al.* 2006; Semenov *et al.* 2007) in manure or manure-amended soil. In addition, Semenov *et al.* (2007) reported that the decline of the pathogen was high when the temperature was increased from 7 to 33°C. On the contrary, Jiang *et al.* (2002) reported a reduced survival at 5°C when compared with 15 or 21°C. In general, for each 10°C increase in soil temperature, the die-off rates of bacterial pathogens are doubled (Leifert *et al.* 2008). In *Salmonella*-contaminated manure stored at different temperatures (4, 20 and 37°C), an exponential reduction in numbers was observed and the main effects of time and temperature were most pronounced, with the most rapid die-off at 37°C (Himathongkham *et al.* 1999).

Generally, more information is needed on behaviour of human pathogens under natural environments and substrates such as manure and soil under variable temperatures on a daily basis. Furthermore, numerous studies indicate that the behavior of bacterial population in soil or manure is complex than the earlier assumptions and involves various processes and interactions with soil biomass, pH ammonium and nitrate content (Zelenev *et al*. 2005). Through greater understanding of the population changes in enteric pathogens under natural conditions we would be better able to predict the risk to human health to foodborne illness in the supply chain.

2.5.3 Soil type

Environmental soils in which *Salmonella* is commonly found are from agricultural fields (Lim *et al*. 2014). Soils of different types differ in their physical, chemical and biological properties. Most of these properties affect the growth and survival of microorganisms in the soil habitat. In general, fine-textured clay soils promote survival of foodborne illness bacteria compared to soils with a coarse sandy texture (van Veen *et al.* 1997; Danyluk *et al.* 2008; Franz and van Bruggen 2008). This is thought to be related to differences in moisture content, WHC, carbon source and oxygen availability. Pore size distribution strongly influences the survival of bacteria in soil and the turnover of organic matter is slower in fine-textured soils than coarser ones (van Veen *et al.* 1997). Studies have reported longer survival of *E. coli* O157:H7 (Ibekwe *et al.* 2004) and *Salmonella* (Natvig *et al.* 2002) in clay-based soil and in soil with higher moisture levels (Mubiru *et al.* 2000). In contrast, *E. coli* O157:H7 survived longer in sandy soil than in clay soil (Ibekwe *et al.* 2007; 2009).

2.5.4 Nutrient status of soil

Nutrient availabilty is a key factor affecting the survival and growth of bacterial pathogens in soil. For example, presence of soil organic matter improves the survival of bacterial pathogens due to increased retention of nutrients, by providing a source of carbon and improving moisture holding capacity (Jamieson *et al.* 2002). Most manure sources are known for their high organic matter content, and it has been reported that the use of fresh manure significantly enhances the survival of *S. enterica* and *E. coli* O157H:7 (Ongeng *et al.* 2015). Manure and manure-amended soil, which have enhanced concentrations of carbon serving as a source of energy, nitrogen and other nutrients, are expected to support survival and growth of bacterial pathogens more than soil with low levels of carbon and nutrients (Ongeng *et al.*

2015). For example, survival of *S.* Typhimurium was significantly increased in manure-amended soil compared to non-amended soil (Garcia *et al.* 2010). Islam *et al.* (2004c) found that soil used to grow parsley and lettuce, which had been amended with composts, supported the survival of *S.* Typhimurium for 232 and 161 days, respectively. In another survival study, *Salmonella* serovars were reported to survive for about 300 days in soil treated with cattle manure (Holley *et al.* 2006).

In addition to carbon and nitrogen, exchangeable ions are also found to affect the survival of *E. coli* O157:H7 in soil. Survival time to reach the minimum detection limit decreased with increasing electrical conductivity and concentrations of individual soil cations (e.g., K^+, Na^+, Ca^+ and Mg^{2+}). These cations interfere with ion transport, enzyme activity and protein synthesis in *E. coli* O157:H7, which in turn result in reduced survival of the pathogen in the soil (Zhang *et al.* 2013).

2.5.5 Soil microbiota

In addition to abiotic stress, enteric bacteria have to compete and interact with endogenous microrganisms to survive within the soil environment (Warriner *et al.* 2009). Some studies indicate that enteric pathogens compete poorly for nutrients and are susceptible to inhibition generated by soilborne bacteria. For example, in manure-amended autoclaved soil, *E. coli* O157:H7 counts increased from 0.3–2.0 $\log_{10}$ CFU g^{-1} within 3 days at 15 and 21°C, whereas the pathogen did not grow in unautoclaved soil likely because of the antagonistic activity of soil bacteria that were present (Jiang *et al.* 2002).

2.5.6 pH

Salmonella spp. can grow in substrates with a pH range from 4.5–9.5 but the optimum pH for most serovars is within the range 6.5–7.5 (Cox and Pavic 2014). Several reports show that pH and soil substrate acidity affected survival of pathogens (Foster and Spector 1995; Lin *et al.* 1996). In general, a neutral pH is expected to favour survival and extreme pH negativley affect the survival of bacterial pathogens (Ongeng *et al.* 2015). While most of the gastrointestinal tract has a neutral pH, *Salmonella* is exposed to acidic pH in the stomach of animal hosts and may develop a mechanism to resist acidic stress (Lin *et al.* 1996). In manure and soil amended with manure, the survival time of *Salmonella* is more strongly affected by temperature less affected by pH (Semenov 2008) and, overall, survival is expected to be

favoured by an acidic pH as the organism seems to have molecular adaptations in response to acidic stresses (Ongeng *et al.* 2015).

2.6 Survival of bacterial pathogens on fresh produce

Even though enteric pathogens are not generally considered to be a part of native plant-bacterial associations, there is emerging evidence that they can to grow and persist both externally and internally on edible plants (Harris *et al.* 2003; Brandl 2006; Gomes *et al.* 2009). The points of entry that bacteria utilise to invade plants are stomata, hydathodes, nectarthodes, lenticels, germinating roots including points where lateral roots arise (Gomes *et al.* 2009).

The ability of pathogens to adhere to fresh produce depends on intrinsic and extrinsic factors including motility of the pathogen, the total microbial environment of the host plant and interaction with these organisms, and availability of nutrient exudates from the plant (Aruscavage *et al.* 2006). The ability of the pathogen to move promotes pathogen entry into openings or wounds on the plant. For example, *S.* Seftenberg has been reported to attach to leafy greens such as basil, lettuce, rocket and spinach, and it is believed that the bacterial motility play a major role in these interactions (Berger *et al.* 2009). *Salmonella* are not attracted to open stomata that are not producing sugar, however the presence of nutrients may attract bacteria to the stomata openings during photosynthesis (Kroupitski *et al.* 2009). It has been reported that the most common area of bacterial aggregation on plants is the area near the trichomes, within the stomates and near veins in the leaves (Aruscavage *et al.* 2006). These regions have high wettability which promotes water availabilty and nutrient leaching that, in turn, support microbial growth.

Pathogen internalisation may be transient and is affected by plant maturity and maturation rate. For example, *S.* Newport invaded aerial sections of romaine lettuce through the roots in 33 day-old plants but not in 17–20 day-old seedlings (Bernstein *et al.* 2007b). However, internalisation of enteric pathogens was mainly observed in seedlings rather than in mature plants (Warriner *et al.* 2003a; Bernstein *et al.* 2007a). Major points of microbial entry include wounds that naturally occur as roots emerge and branch creating natural entry points (Warriner *et al.* 2003a). A three-phase process of plant internalisation has been proposed in roots of hydroponically-grown tomato plants infected with *Ralstonia solanacearum*: (1) colonisation of the root surface and formation of microcolonies at locations with nutrient

leakage; (2) infection of the vascular parenchyma as the population number reaches a level where plant defense is no longer effective; and (3) invasion of the xylem (Klerks *et al.* 2007a). There may be similar mechanisms of entry for *Salmonella* into tomato roots to that of *R. solanacearum* (Schell 2000; Klerks *et al.* 2007a) however this has not been confirmed (Schell 2000).

Many studies have indicated the internalisation of human pathogens in or on plants. *Escherichia coli* O157:H7, *Salmonella* and *L. monocytogenes* have all been found to colonise various vegetables at different growth stages and in different tissues (Table 2.6). For instance, *Salmonella* and *L. monocytogenes* can be internalised within core tissue of tomatoes and in the stomata of lettuce (see Figure 2.3) and spinach (Zhuang *et al.* 1995; Ölmez and Temur 2010). In another study, *S.* Typhimurium was found to colonise roots of carrot and radish, grown under field conditions treated with contaminated manure, compost or irrigation water (Islam *et al.* 2004b). *Escherichia coli* O157:H7 has been reported to enter lettuce plants through the root system and move via the vascular system to the leaves above ground (Solomon *et al.* 2002).

Table 2.6 Internalisation of pathogens within growing plants. Adapted from Warriner *et al.* (2009), Fornefeld *et al.* (2017) and Nicholson *et al.* (2015).

Pathogen	**Plant**	**Reference**
Escherichia coli O157:H7	Radish sprouts	Itoh *et al.* (1998)
Salmonella	Tomato stems and blossoms	Guo *et al.* (2001)
E. coli O157:H7	Lettuce seedlings	Solomon *et al.* (2002)
Nonpathogenic *E. coli*	Cabbage seedlings	Rafferty *et al.* (2003)
E. coli O157:H7 and *Salmonella*	*Arabidopsis* plants	Cooley *et al.* (2003)
E. coli	Spinach plants cultivated in soil or hydroponically	Warriner *et al.* (2003a)
E. coli and *Salmonella*	Mung bean sprouts	Warriner *et al.* (2003b)
E. coli O157:H7, *Salmonella* and *Listeria monocytogenes*	Various leaf vegetables	Jablasone *et al.* (2005)
Salmonella	Parsley stomata and cut cuticle cracks	Duffy *et al.* (2005)
Salmonella	4 week-old barley roots	Kutter *et al.* (2006)
E. coli O157:H7	Mature lettuce	Bernstein *et al.* (2007a)

Pathogen	Plant	Reference
E. coli O157:H7 and *Salmonella*	Hydroponically cultivated lettuce	Franz *et al.* (2007)
Salmonella	Tomato blossoms	Shi *et al.* (2007)
E. coli O157:H7 and *Salmonella*	Romaine lettuce	(Nicholson *et al.* 2015)
Salmonella	Lettuce	(Zhang *et al.* 2016)

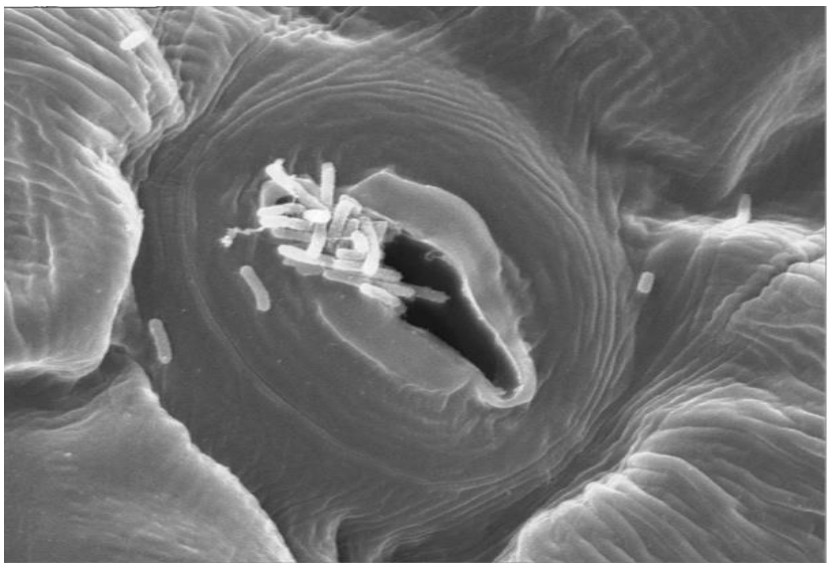


Figure 2.3 Scanning electron microscopy image showing the complex topography of a single stomatal region and multiple bacteria (potentially *Salmonella*) internalising a lettuce leaf within the stomatal space. From Kroupitski *et al.* (2009).

In contrast to these studies, Fatica and Schneider (2011) reported that *Salmonella* spp. lack the adaptability to survive on plants. Rather, the pathogen attaches to the plant as part of a biofilm (Figure 2.4) which enable the bacteria to persist on or within the plant. Biofilms protect bacterial cells from adverse environmental conditions such as low humidity and bactericidal agents such as sanitisers, commonly used in the industry (Buck *et al.* 2003; Morris and Monier 2003).

Upon attachment of *Salmonella* to leaf surfaces, bacteria exposed to environmental conditions such as temperatures below 30°C and low levels of atmospheric oxygen may trigger expression of regulatory sRNAs and proteins (e.g. RpoS, CsgD and SirA) (Yaron and Römling 2014). Stress signals that are a result of low nutrient availability and the presence of antimicrobial compounds produced by the plant or indigenous microorganisms can also

enhance the expression of sRNAs and proteins. The genes involved in production of components responsible for the biofilm matrix such as cellulose, curli and BapA are activated by stimulation of regulatory proteins leading to the development of biofilms on the leaf surface. The biofilm components contribute to the induction of local and systemic plant defense responses while the biofilm structure stabilises bacterial colonisation on the plant and provide protection from various stresses (Yaron and Römling 2014).

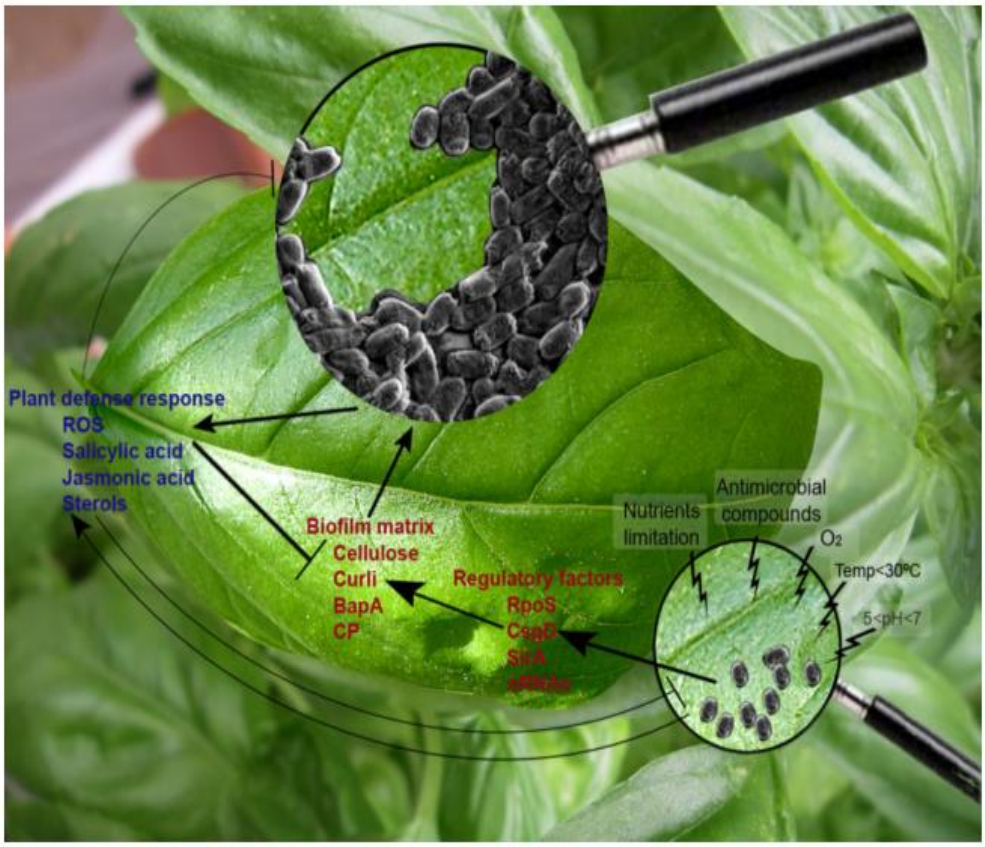


Figure 2.4 Biofilm formation on leaves by *Salmonella*. From Yaron and Römling (2014).

Many studies indicate that after successful attachment or internalisation, human enteric pathogens may survive for long periods. In the field, *E. coli* O157:H7 and *Salmonella* were detected after 177 and 231 days, respectively, on parsley plants (Islam *et al.* 2004a; c). In addition, 17 weeks after the application of contaminated manure, *S.* Typhimurium was isolated from arugula plants after harvesting (Natvig *et al.* 2002). However, the long term survival of microorganisms on fresh produce is affected by nutrient availability, the presence of toxic compounds generated by the plant and antagonistic activity from other microorganisms (Whipps *et al.* 2008).

Some studies report that the internalisation of human enteric pathogens within plant tissue is not through root hairs; rather, possible contamination of vegetables growing in contaminated soil is through splash from irrigation or rain (Natvig *et al.* 2002). *Escherichia coli* O157:H7 was not internalised within intact lettuce leaves and roots after microbial inoculation of the leaf surface and soil regardless of the type of lettuce, age of plants or strain of bacteria in more than 500 surface-sanitised lettuce leaves and roots (Zhang *et al.* 2009). Only two of the root samples were positive and these two positive samples were likely to have resulted from inadequate surface sanitising or infiltration of *E. coli* O157:H7 through wounds. In similar studies, *E. coli* O157:H7 was not able to colonise spinach plants (Hora *et al.* 2005) or crisp head lettuce (Johannessen *et al.* 2005). It is evident that plant internalisation by enteric pathogens, either through root hairs, wounds or stomatal openings, is not well described in the literature and requires further research.

2.7 Control measures for bacterial pathogens on fresh produce

Current public health concerns regarding microbiological contamination of fresh produce has focused research on reducing or minimising the risk of contamination throughout the production system from the paddock to the plate but particularly during the postharvest washing stage (Franz and van Bruggen 2008). Sanitisers such as chlorine, chlorine dioxide, bromine, acidified sodium chlorite, iodine, alkaline compounds, organic acids, hydrogen peroxide, ozone and irradiation are commonly used (Franz and van Bruggen 2008). In addition, preharvest measures may be undertaken to prevent the risk of contamination of fresh produce in the field.

2.7.1 Postharvest measures

Postharvest washing of produce presents an opportunity for cross-contamination to occur so sanitisers are often used, with chlorine being the most common. The use of peroxyacetic acid for sanitising specific food products, including fresh produce, at concentrations that do not exceed 80 ppm in wash water has been approved in the USA (Olaimat and Holley (2012). Regulation of sanitiser use vary from country to country and some may be prohibited in organic production (Franz and van Bruggen 2008).

It may be difficult to clean fresh produce once it is contaminated because the efficacy of sanitisers in removing *Salmonella* depends on its location on the produce. *Salmonella* on stem scars and cracks in the skin survive better than *Salmonella* on smooth skin (Hanning *et al.*

2009). Furthermore, the ability of *Salmonella* and other foodborne pathogens to internalise creates a significant challenge in eliminating bacteria from these foods. Chlorine and treatments such as electrolysed water or ozonated water may eliminate *Salmonella* from the surface of fresh produce but are ineffective at reaching at any internalised plant parts (Chaidez *et al.* 2007; Park *et al.* 2008). These treatments targeted at enteric pathogens also remove and kill native microflora which might otherwise have an inhibitory effect on pathogens by competing directly for space and nutrients or by producing antagonistic compounds.

Investigations have shown that ultraviolet (UV) light is effective in reducing microorganisms in food products; however, applications remain limited because the poor penetration of UV radiation into food matrices can make treatments unreliable. The effectiveness of UV light decreases as the amount of suspended particles in the treatment medium increases. In addition, some bacteria exhibit enhanced resistance to UV-C when in the stationary phase of growth (Child *et al.* 2002; Bucheli-Witschel *et al.* 2010). Some authors have suggested that a greater reduction in microbial loads can be achieved by combining UV light with heating to temperatures that are lower than those used in pastuerisation (Gayán *et al.* 2012). A drawback associated with use of UV radiation in foods is that high levels of irradiation can produce off-flavours and discolouration whereas lower levels may not be fully effective in eliminating bacteria (Hanning *et al.* 2009).

2.7.2 Preharvest measures

2.7.2.1 Composting practices

Generally, composting of manure before application to soil is required to decrease the risk of bacterial pathogens. This practice helps to reduce the level of pathogens before application to agricultural fields which, in turn, minimises contamination of soil, surrounding water and produce (Islam *et al.* 2004b; Ceustermans *et al.* 2007). Several factors affect the composting process such as pH, particle size, porosity, nutrient balance, temperature and water content. A high carbon:nitrogen (C:N) ratio makes the composting process slow as there is an excess of degradable substrate as an energy source for the microorganisms. With low C:N ratios, there is an excess of N per degradable carbon and inorganic N which is produced in excess, can be lost in the form of ammonia or nitrate. Compost with a low C:N ratio can be corrected by adding a bulking agent such as straw to provide degradable organic carbon.

Proper aeration is an important factor in the composting process as it controls temperature, removes excess moisture and CO_2 and provides O_2 for respiration of aerobic microbes (Bernal *et al.* 2009). The optimum temperature range for composting is 40–65°C and temperatures above 55°C are required to kill pathogenic microorganisms (Bernal *et al.* 2009). According to Manyi-Loh *et al.* (2016), the threshold levels of *Salmonella* after a composting process is <3 most probable number (MPN) g dry matter^{-1} in the USA, 0 per 25 g wet weight^{-1} in France, <100 MPN g dry matter^{-1} in Italy and 0 per 25 g fresh mass^{-1} in the United Kingdom. For fecal coliforms, the maximum level is <1000 MPN g dry matter^{-1} in the USA and Canada.

Bacterial pathogens are occasionally detected in compost products (Pera *et al.* 1991; Gong *et al.* 2005; Gong *et al.* 2006). In the United States, a survey of 72 commercial composting facilities found that more than half of the facilities were positive for *Salmonella* spp. Some of the products were contaminated despite meeting the time-temperature criteria for decontamination (Hay 1996). In an experiment on survival of bacterial pathogens in composted products, coliform bacteria and *Salmonella* were detected in 37 and 16% of samples, respectively (Gong *et al.* 2005; Elving 2009). Pathogenic bacteria in most samples were destroyed by the composting process; however, some pathogenic bacteria survived, especially in compost derived from organic refuse. This is because the organic waste provides a carbon source which enables the pathogen to survive for long periods of time (Gong *et al.* 2005; Elving 2009). Three categories of compost products: (i) raw material held at 48°C for 20 days; (ii) uncompleted compost held at 45°C for 30 days; and (iii) compost product held at 44°C for 50 days were found to contain more than 10^7, 3.7 x 10^4 and 6.0 x 10^3 CFU g^{-1} of *Salmonella*, respectively (Gong *et al.* 2005). In addition, it was reported that *Salmonella* and *E. coli* survived for 95 days at 60°C in industrial compost (Droffner and Brinton 1995). These studies indicate that a number of pathogenic bacteria can survive even after being exposed to high temperatures for long periods (Gong *et al.* 2005).

According to the USDA organic certificate program, harvesting a crop that does not touch the soil, may not occur inside a window of 90 days after addition of manure into the soil. For crops that do come in contact with the soil, the rule states at least 120 days should elapse after manure addition, before harvesting an edible product (Anonymous 2000, as cited in Franz and van Bruggen 2008). In the United Kingdom, 6 months is recommended between the time of manure application and harvest (Nicholson *et al.* 2000). According to Australian and New Zealand guidelines for food safety of fresh produce, the exclusion period between the

application of untreated manure and crop harvest, particularly for lettuce is 90 days (Fresh produce safety center 2015). In many countries such regulations do not exist and uncomposted manure is still widely used in vegetable production (Semenov 2008).

2.7.2.2 Soil biofumigation

Soil pests and pathogens may be suppressed or killed by naturally occurring biocidal compounds released after a green manure crops is incorporated into the soil and is termed biofumigation (Kirkegaard *et al.* 1997; Matejiceck *et al.* 2002; Gimsing *et al.* 2005). The active compounds are typically secondary plant metabolites such as glucosinolates and phenolics.

Glucosinolates (GSLs) are a group of compounds produced by the family Brassicaceae (such as broccoli, cabbage, kale, mustard and canola); however, they are not confined to this family (Fahey *et al.* 2001). The properties of GSLs and their degradation products were first observed at the beginning of the 17th century while investigating the reason for the sharp taste of mustard seeds (Challenger 1959). When tissues of GSL-containing plants are damaged or disrupted, GSL comes into contact with the endogenous enzyme myrosinase, which hydrolyses the GSL to several biologically active compounds (Delaquis and Mazza 1995; Mari *et al.* 2008; Al-Gendy *et al.* 2010). Even though there are more than 120 different side chains, GSLs are categorised in three groups: aliphatic, aromatic and indolyl (Gimsing *et al.* 2005). The differences in chemical properties, biological activity and the hydrolysis products of the various GSLs are largely determined by the side chains (Gimsing *et al.* 2005). Glucosinolates are polar and highly water-soluble compounds and when they come in contact with myrosinase they hydrolyse relatively quickly, especially if there is water present, with isothiocyanates (ITCs) the most common hydrolysis products (Wathelet *et al.* 2004).

Concentrations of GSLs vary in different plant species and tissues. For example, in canola, the mature aboveground plant tissue has very low concentrations, whereas the roots are the principal source of GSLs for biofumigation (Kirkegaard *et al.* 1997).

There are many processes following the release of GSLs into soil which influence the effectiveness of suppression (Kirkegaard *et al.* 1997). Some mechanistic studies have indicated the antibacterial effects of ITCs even though their mode of action is still unclear. This might be through damaging cellular structures, particularly the plasma membrane, which led to ATP leakage in *E. coli* (Lin *et al.* 2000; Luciano and Holley 2009). It has been reported

that Gram-negative bacteria tend to be more sensitive to ITCs than their Gram-positive counterparts (Wilson *et al.* 2013). Preliminary studies in north Queensland, Australia indicated that the population of *Ralstonia solanacearum*, the bacteria responsible for bacterial wilt, declined from 10^7 CFU g^{-1} soil to undetectable levels after incorporation of canola residue and incubation for 4 weeks (Akiew and Trevorrow 1997, as cited in Kirkegaard *et al.* 1997).

Many studies have proposed that phenolic compounds from various plants may also have biofumigation effects against pathogenic bacteria (Cetin-Karaca 2011). For instance, phenolic compounds extracted from mushrooms had antimicrobial activity against both Gram-positive and Gram-negative bacteria (Alves *et al.* 2013). Protocatechuic acid, 2,4-dihydroxybenzoic acid, vanillic acid, *p*-coumaric acid and cinnamic acid derivatives are some common phenolic compounds shown to possess antimicrobial activity (Alves *et al.* 2013). For example, *p*-coumaric acid has an antimicrobial effect against Gram-negative bacteria such as *E. coli*, *S.* Typhimurium and *Shigella dysenteriae* through changing the permeability of the cell membrane. It also has the capacity to bind to DNA, inhibiting general cell function (Lou *et al.* 2012). Chestnut and mimosa tannins were found to reduce the growth rate of *E. coli* O157:H7 with the degree of growth reduction correlated with tannin concentration (Min *et al.* 2007). Syringic and ellagic acids (0.5 mg mL^{-1}) have an antimicrobial effect against *L. monocytogenes*, while cinnamic acid inhibits *Streptococcus agalactiae* at the same concentration (Alves *et al.* 2013). In another study, the rate of death of *E. coli* O157:H7 was found to increase 17-fold and 23-fold by the addition of 0.5% trans-cinnamic acid or 0.5% para-coumaric acid, respectively, compared to control treatments with no addition (Wells *et al.* 2005).

2.7.2.3 Soil solarisation

Soil solarisation is a non-chemical approach (Stapleton and DeVay 1986; Raio *et al.* 1997) which is commonly used to treat soil to reduce microbial activity (Meays *et al.* 2005). Solarisation is achieved by covering the soil with plastic film to retain solar heat energy, thereby raising the soil temperature to levels that are lethal to many pathogens (Gamliel and Stapleton 1997). Solarisation is usually applied for 4 weeks or longer to suppress soil pathogens to depths of 45–60 cm below the soil surface. For example, heating the soil to a temperature of 45°C to simulate solarisation was very effective in reducing the viability of *Pythium ultimum* and *Sclerotium rolfsii* (Gamliel and Stapleton 1997). Soil temperature

during solarisation is low compared to artificial heating methods such as steaming. There are side effects with soil steaming such as phytotoxicity and pathogen reinfestation due to the creation of a biological vacuum; however, this has not been reported with solarisation (Gamliel and Stapleton 1997).

2.7.2.4 Biofumigation with solarisation

In a soil solarisation experiment where soil was amended with cabbage residue, it was reported that the concentration of volatile compounds was higher in heated (solarised) soil than in the corresponding unheated soil (Gamliel and Stapleton 1993). The addition of broccoli residue to soil at 20°C was not effective in supressing root gall in melon plants; however, temperatures of 30–35°C for a period of 10 days almost eliminated the galling on the roots after soil amendment with broccoli residues (Ploeg and Stapleton 2001). In a similar soil solarisation study, the number of propagules of *Pythium ultimum* and *Sclerotium rolfsii* were reduced by more than 95% when the propagules were exposed to volatile compounds generated from heated cabbage-amended soil but the propagules from both fungi were not reduced to this extent when exposed to compounds generated from unnheated cabbage-amended soil (Gamliel and Stapleton 1993). Other studies involving the combination of biofumigation with solarisation have been done for the control of, among other pests and diseases, root-knot nematodes (Moura *et al*. 2012), bacterial spot (Misrak *et al*. 2014) and *Phytophthora* (Poras *et al*. 2009) but there are no recent studies using both treatments for control of foodborne pathogens.

2.8 Summary

Foodborne illness is a serious problem that can affect large numbers of people. The negative publicity associated with food recalls reduces consumer confidence and product demand leading to substantial economic losses for all sectors of the supply chain. It is imperative that all parts of the supply chain are dedicated to implementing good management practices that reduce the risk of contamination (Berger *et al.* 2010). There is a significant amount of research into development of approaches to eliminate or suppress enteric pathogens in both preharvest and postharvest stages.

Many postharvest measures are employed to suppress enteric pathogens associated with fresh produce. However, sanitising agents may be ineffective in eliminating pathogens from the produce due to internalisation, formation of biofilms or other mechanisms. Therefore,

preharvest measures to remediate contaminated soil are critical in maintaining the safety of fresh produce. Soil biofumigation and solarisation have been found to suppress some fungal and bacterial pathogens in the soil habitat but more research is required in this area.

3. General materials and methods

3.1 Site description

The two soils used in the microcosm pot trial (Chapter 4) and field experiment (Chapter 5) were collected from two properties owned by the University of Sydney. Coarse sand (hereafter referred to as 'sandy soil') was collected from Karalee Farm near Camden, from an area which had previously been used as pasture and for small scale horticultural crop production. The site is located approximately 70 km from Sydney (34°00'57.90" S, 150°40'19.17" E). The predominant soil type is classified as a Rudosol and the climate is characterised by warm-to-hot summers and cool-to-mild winters. The annual mean maximum and minimum temperatures in this area are 23.7°C and 10.2°C, respectively, with the highest and lowest mean temperatures occurring in the months of January and July, respectively. Rainfall is approximately 788 mm per annum (Bureau of Meteorology 2016a).

Clay loam (hereafter referred to as 'clay soil') was collected from John Bruce Pye Farm (hereafter referred to as Pye Farm), which is a research facility used for large-scale cereal breeding and fungicide trials. Pye Farm is located near Bringelly, approximately 60 km from Sydney (33°56'33.92" S, 150°40'06.02" E). The area is dominated by red Sodosol soils and the climate is also characterised by warm-to-hot summers and cool-to-mild winters. The annual mean maximum and minimum temperatures in the area are 23.8°C and 10.8°C, respectively, with the highest and lowest temperatures in the area occurring in the months of January and July, respectively. Rainfall is approximately 750 mm per annum (Kehlet 2010; Bureau of Meteorology 2016b).

The two soil types (clay and sandy) were selected to evaluate the die-off of *Salmonella* in two contrasting soil types under Australian conditions. The soils were exposed to similar conditions such as temperature, moisture and incubation time for the microcosm pot trial whereas for the field experiment, they experienced similar ambient environmental conditions, except for date of sampling and irrigation interval.

3.2 Soil description

3.2.1 Microcosm soil pot trial

Bulk soil used for the microcosm pot trial was sieved to 2 mm and stored until required. For preparation of samples for soil analysis, the same masses of soil used in the microcosm pot study (see Chapter 4) were placed in incubation vials and chicken manure was added at the same rate as that used in the microcosm pot trial (i.e. 0 or 2%, w/w; n = 5 for each treatment). Phosphate buffer solution was added (5 mL) to each replicate to imitate addition of inoculum in the original microcosm pot trial (see Chapter 4). It was presumed that soil moisture was similar as in the original study (i.e. 15.2 ± 0.8% for clay soil and 9.6 ± 0.5% for sandy soil). The soil was allowed to equilibrate for 48 h at 5°C prior to analysis.

3.2.2 Field experiment soil

Soil samples were randomly taken using soil cores from 10 points across each field (5 cm diameter, 10 cm depth), sieved to 2 mm, air dried and bulked to form a single composite sample.

3.2.3 Soil analyses

The chemical and physical properties of soil from both Karalee Farm and Pye Farm, used for the microcosm pot trial and field experiment, were analysed as described below. Some analyses (such as soil colour, phosphorus, potassium, sulphur and micronutrients) were performed by an analytical services provider accredited by the Australasian Soil and Plant Analysis Council. The methods for these analyses are not described in this chapter.

3.2.3.1 Particle size analysis

Soil texture was determined using the standard hydrometer method (Bouyoucos 1962). Approximately 50 g of soil from the composite sample was shaken with 50 mL of 5% sodium hexametaphosphate solution (at pH 8.5) for 48 h. After transferring the soil mixture into a large measuring cylinder, a dilution was made up to 1 L using deionised water and stirred thoroughly using an agitator. A hydrometer was carefully immersed into the soil mixture and a reading was recorded at 5 min and 8 h. After all measurements were taken, the suspended clay particles were discarded and the material that had settled was washed by repeating the process of stirring with water and decanting the suspended material at least five times.

To remove any organic matter, the remaining soil mixture was treated using 30% hydrogen peroxide solution. After adding 25 mL of hydrogen peroxide (H_2O_2) to the soil mixture, it was swirled to mix thoroughly. The mixture was then heated at 60°C for 2 h. When effervescence occurred, the beaker was removed from the heat until the reaction ceased. More H_2O_2 (5 mL aliquots) was added to the soil mixture and the process was repeated until there was no further effervescence. The sample remaining at the end of the treatment was sieved to fractions greater than 100 µm and less than 100 µm. Both fractions were oven-dried at 105°C for 24 h and dry weights recorded.

3.2.3.2 Total carbon and nitrogen

Composites soil samples were ground (53 µm) and analysed for total nitrogen (N) and carbon (C) by dry combustion (Elementar, CN Analyzer, Germany).

3.2.3.3 Ammonium and nitrate

Soil extracts were prepared by shaking 5 g of fresh soil with 0.05 M potassium sulphate for 1 h then filtered with ashless filter paper. Using a continuous flow injection system (QuikChem®, Lachat Instruments, USA), ammonium and nitrate were determined colorimertically based on the standard Lachat method (Hofer 2003; Pritzlaff 2003). To describe this process briefly, soil extracts were mixed with salicylate and hypochlorite in an alkaline phosphate buffer and heated gently. Ammonium in soil extracts was determined by colorimetric analysis at 630 nm. For the determination of nitrite, an aliquot of the soil extract was passed through a copperised cadmium column. Nitrate in soil extracts was reduced to nitrite, which was then measured by diazotising with sulphanilamide and mixed with N-(1 naphthyl) ethylenediamine dihydrochloride. The concentration of nitrate was determined colorimetrically at 520 nm.

3.2.3.4 Gravimetric moisture content

15 g soil samples from the bulk soil used for microcosm pot trial and from soil collected from the field (see above) were used to determine moisture content. The weight of soil was recorded before and after oven-drying at 105°C for 24–48 h. Gravimetric moisture content (%) was calculated as:

$$\text{Gravimetric moisture content} = \frac{\text{Soil wet weight (g)} - \text{soil dry weight (g)}}{\text{soil dry weight (g)}}$$

3.2.3.5 Soil acidity and electrical conductivity

Electrical conductivity (EC) and pH of soil was measured in a water suspension of 1:5 soil:H_2O. Approximately 5 g of field moist soil was mixed with 25 mL deionised water and shaken on a wheel rotator for 15 min. Samples were allowed to settle for 15 min before measurement. Values for pH and EC in the water suspension were measured using a pH meter (PHM210, MeterLabTM) and an EC meter (CDM210, MeterLabTM), respectively.

3.2.3.6 Bulk density

Soil samples for measurement of bulk density were taken from an undisturbed horizontal surface after removal of any plant or dead material from the soil surface. A steel core (5 cm diameter, 10 cm depth) was hammered into the soil and excavated without disturbing the soil within the core. The core was carefully removed keeping the soil intact and any excess soil from the outside ring was removed. The soil was transferred to a plastic bag and sealed to prevent water loss. The soil was oven dried for 48 h at 105°C and dry weight was measured for clay soil (Pye Farm) and sandy soil (Karalee Farm). Soil bulk density was calculated using the following formula (n = 5 per site):

$$\text{Bulk density (g cm}^{-3}\text{)} = \text{Dry soil weight (g)/ soil volume (cm}^{3}\text{)}$$

3.3 Preparation of media

All basal growth media were prepared as per manufacturer instructions (see Appendix 1). These include nutrient agar (NA), Trypticase soy agar (TSA), Tryptic soy broth (TSB), Xylose lysine deoxycholate (XLD) agar, Xylose-lysine-tergitol 4 (XLT4) with agar supplement, phosphate buffer, mBroth, Demi-Fraser broth, and *Listeria* chromogenic agar. The modified media which were prepared by adding sodium pyruvate, iodine supplement and rifampicin are presented below.

3.3.1 Preparation of trypticase soy agar with sodium pyruvate and rifampicin

Rifampicin (32 mg) (Biochemicals, Australia) was dissolved in 1 mL of methanol and the solution was sterilised by passing through a sterile 0.22 µm syringe filter into a sterile centrifuge tube. The stock solution was covered with aluminum foil and stored at 5°C until required.

Trypticase soy agar (TSA) medium was prepared by suspending 16 g dehydrated TSA and 0.4 g sodium pyruvate (Sigma-Aldrich, USA) in 400 mL distilled water in a bottle and boiled until completely dissolved. Sodium pyruvate is added to bacterial media as an additional source of energy and it also enhances the recovery of the bacterial cells (Gamer and Elsanousi 2016). The medium was sterilised by autoclaving at 121°C for 20 min, then allowed to cool to approximately 50°C. Sterile rifampicin (1 mL) was added to the medium and mixed using a magnetic stirrer with a sterile stirrer bar prior to pouring into Petri dishes, which were stored at 5°C until required. Rifampicin plates were stored in the dark for no longer than 14 days to prevent light-mediated degradation of rifampicin prior to use. Trypticase soy agar plates amended with rifampicin are abbreviated as TSARP throughout the thesis.

3.3.2 Preparation of xylose lysine deoxycholate agar with sodium pyruvate and rifampicin

The XLD medium was prepared by suspending 22 g dehydrated XLD agar and 0.4 g sodium pyruvate in 400 mL distilled water. The medium was heated to boiling as described in Appendix 1. The medium was cooled to approximately 50°C and sterile rifampicin was added to the medium prior to pouring plates.

3.3.3 Preparation of *Salmonella* extraction media

An extraction solution comprising 0.02 M sodium phosphate solution and 0.1% Tween 20 was prepared by mixing 38 g sodium phosphate (Chem-Supply, Australia) and 5 mL Tween 20 (EMD Millipore, USA) in 5 L distilled water. The medium was sterilised by autoclaving at 121°C for 20 min and cooled to room temperature before storage at 5°C.

3.3.4 Preparation of buffered peptone water

Buffered peptone water (BPW) was used as pre-enrichment media for detection of low numbers of *Salmonella* in soil samples. This solution was prepared by suspending 20 g of dehydrated media (Difco™, BD, Sparks, USA) in 1 L distilled water. The medium was heated on a hot plate with a magnetic stirrer until completely dissolved and then sterilised by autoclaving at 121°C for 20 min. Double strength BPW was prepared by suspending 40 g of dehydrated media in 1 L distilled water and sterilised by autoclaving at 121°C for 20 min.

3.3.5 Preparation of tetrathionate broth base

Tetrathionate broth base (TTB) with iodine solution was used as a selective enrichment medium for the isolation of *Salmonella*. The TTB was prepared by suspending 18.4 g of dehydrated medium (Difco, BD, Sparks, USA) in 400 mL distilled water. The medium was heated to boiling (using a 240 W microwave at medium power for 2 min; high power for 2 min; medium power for 2 min) with frequent agitation to dissolve completely without autoclaving or overheating the medium. The broth base was stored (without iodine solution) in the dark at 5°C and 8 mL of iodine supplement was added to 400 mL of TTB broth and used immediately.

The iodine supplement was prepared by dissolving 6 g iodine and 5 g potassium iodide in 20 mL distilled water. The solution was stored in tightly-sealed amber vials or wrapped in foil due to light sensitivity. The solution was sterilised by passing through a sterile 0.22 µm syringe filter into a sterile tube and stored at room temperature.

3.4 *Salmonella* serotyping

3.4.1 Slide agglutination test

Salmonella cultures in this study were serotyped using O-antisera (Statens Serum Institut, Denmark) as a confirmatory procedure, since known serovars were being used for inoculum preparation (Table 3.1). A colony was isolated from non-selective NA or TSA agar plates and smeared on a glass slide containing a drop of antiserum. The slide was rocked back and forth for 1 min and observed for agglutination, indicating presence of the specific antigen (Figure 3.1).

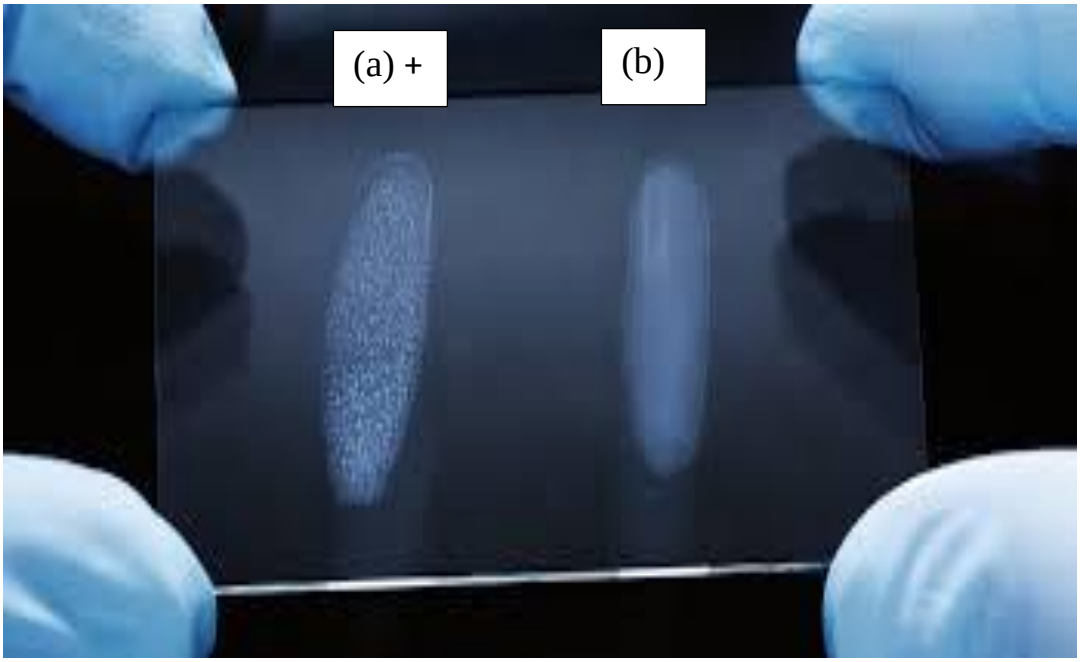


Figure 3.1 Slide agglutination test showing a positive (a) and negative (b) result for *Salmonella* samples.

Table 3.1 *Salmonella* serovars used in the present study and their antigens. Adapted from Brenner *et al.* (2000), Grimont and Weill (2007) and Hendriksen *et al.* (2009).

O group	Serovar	O-antigens	H-phase 1 antigens	H-phase 2 antigens
B	*S.* Typhimurium	1,4,[5],12	i	1,2
B	*S.* Sofia	1,4,12,27	b	e, n, x
C1	*S.* Infantis	6,7,14	r	1,5
C1	*S.* Montevideo	6,7,14	g,m,[p],s	[1,2,7]
D	*S.* Enteritidis	1,9,12	g,m	No phase 2 antigen
E	*S.* Zanzibar	3, {15}	k	1,5

3.5 Experimental cultures

3.5.1 Experimental cultures used

Because of their role in foodborne disease outbreaks associated with poultry and fresh produce, *Salmonella* serovars Enteritidis, Infantis, Montevideo, Sofia, Typhimurium and Zanzibar were used in the present study (kindly donated and verified by Birling Avian

Laboratories, New South Wales, Australia). The serovars were maintained as glycerol (15% + TSB) stock cultures at -80°C after undergoing serological testing.

S. Enteritidis and *S.* Typhimurium, which are used in the present study, are most common serotypes in foodborne illnesses. The third serovar used in this study was *S.* Sofia, which is the most prevalent serovar in Australia, though it is not pathogenic in humans. It is a geographically unique bacterial species that is consistently and almost solely isolated in Australia, mostly from poultry and poultry products (Mellor *et al.* 2010). *S.* Sofia and *S.* Typhimurium belong to the same O group (Table 3.1), however, the former is avirulent whereas the latter is virulent. The fourth serovar used in the present study, *S.* Infantis, is found to be a prevalent serovar within the poultry industry worldwide. *S.* Infantis is associated with the egg and the chicken meat industry and has been of important public health concern in many countries and remains so in some areas (Cox *et al.* 2002). The fifth serovar used was *S.* Montevideo, which was found to be among the top five serovars causing *Salmonella* infection in Australia in 2008–2009 (OzFoodNet Working Group 2010). The fifth serovar used was *S.* Zanzibar, which was reported as a cause of foodborne disease outbreaks in New South Wales, Australia in 2013.

3.5.2 *Salmonella* culture maintenance

To prepare frozen *Salmonella* cultures, pure serotyped *Salmonella* serovars were streaked on TSA plates and incubated at 37°C for 24 h. Using a sterile loop, five single colonies from the TSA plate were used to inoculate 5 mL sterile TSB (thoroughly mixed) and incubated at 37°C for 24 h. After checking the bacterial growth, which is characterised by cloudy suspension in the media, filter-sterilised glycerol (15% v/v) was thoroughly mixed with the fresh *Salmonella* broth culture in a 2 mL cryovial. Duplicate stock cultures were prepared for each serovar and all stock cultures were stored at -80°C.

To prepare a working culture, a small amount of the frozen culture was removed using a sterile loop (1 µL), streaked onto XLD agar and incubated at 37°C for 24 h. After incubation, a single colony was isolated, streaked on XLD plates and incubated at 37°C for 24 h. A single colony was picked from the XLD plates, streaked on TSA and *Salmonella* CHROMagar plates and incubated at 37°C for 24 h. Mauve-coloured colonies on CHROMagar were presumptively identified to be *Salmonella* and further confirmation was performed using serological tests as described in 3.4.1.

The serotyped pure culture was used as a working culture which was subsequently subcultured on a monthly basis. TSA or NA plates were the preferred medium for short-term storage (<4 weeks) at 4°C. Pure *Salmonella* cultures were also grown and maintained on NA slants for medium-term storage (<6 months).

3.6 Experimental procedures

3.6.1 Inoculum preparation

A working bacterial culture was prepared as described in Chapter 3.5.2. After serotyping, TSA plates were used to prepare a *Salmonella* suspension in sterile phosphate buffer. The suspension was thoroughly mixed for 1 min using a vortex-mixer. To prepare a lawn culture, 100 µL of the suspension was spread on TSA plates and incubated at 37°C for 24 h. The resulting bacterial lawn was collected by adding sterile phosphate buffer to each plate, loosening the lawn with a sterile spreader and transferring the suspension into a sterile container. After preparing serial 10-fold dilutions using sterile phosphate buffer, the concentration of the bacterial cells in the inoculum was measured by plating 50 µL on replicate XLD plates using a spiral plater (easySpiral®, Interscience, France). Streaking of the same samples was performed on TSA plates and incubated at 37°C for 24 h. Sample purity was checked using specific antiserum from TSA plates and the inoculum was then ready to inoculate the soil or chicken manure samples.

3.6.2 Extraction and enumeration of *Salmonella* from soil or chicken manure samples

Soil samples (100 g) obtained from the two sites (as described in Chapter 3.2) or chicken manure (CM) obtained from a commercial market (Enfield Produce, Pet and Garden Supplies, Sydney, Australia) were aseptically transferred to a 400 mL sterile stomacher bag (BagSystem®, Interscience, France) using a sterile spoon. The weight of the sample was measured by gravimetric dilutor (BabyGravimat®, Interscience, France) and 100 mL (for microcosm pot trial) and 150 mL (for field experiment) of extraction solution (as described in 3.3.3) was dispensed into the sample bag by the gravimetric dilutor. The sample was homogenised using a stomacher (BagMixer®, Interscience, France) for 1 min at speed 1 (low). After mixing, the sample was allowed to settle for 15 min. A 5 mL aliquot was aseptically removed using a 5 mL sterile serological pipette and transferred to a sterile microcentrifuge tube for subsequent enumeration. Serial 10-fold dilutions of this aliquot (1 mL) were made using sterile phosphate buffer and an aliquot (100 µL) was spread on XLD

agar plates using a spiral plater. All XLD plates were incubated at 37°C for 24 h. At the beginning and end of plating, sterile phosphate buffer (100 µL) was spread on TSA plates as a quality control check of the spiral plating system.

After incubation, colonies were counted using an automatic colony counter (Scan®500, Interscience, France). Thirty to 300 colonies per plate were considered to be optimal countable numbers. For plates with more than 300 colonies, the original suspension, stored for 24 h at 5°C, was serially diluted with sterile phosphate buffer and plated on XLD agar, incubated at 37°C for 24 h and counted again. Extracts that yielded no *Salmonella* growth (zero count) by direct plating were re-analysed following an enrichment protocol (see Chapter 3.6.3). The limit of detection (LOD) was calculated to be 1.18 $\log_{10}$ CFU g^{-1}.

3.6.3 Enrichment steps for *Salmonella*

A 95 mL aliquot of the original suspension (remaining after taking the 5 mL aliquot for enumeration, Chapter 3.6.2) was enriched with 95 mL of double strength BPW and incubated at 37°C for 24 h. After incubation, a 10 mL aliquot was transferred to 90 mL TTB plus iodine (88.2 mL TTB + 1.8 mL of iodine and potassium iodide) and incubated at 42°C. After 6 h incubation, 10 mL of this solution was transferred to 90 mL mBroth and incubated at 37°C for 24 h. To inhibit the growth of other microorganisms, the mBroth sample was stored at 5°C for 2 days. The mBroth sample was then streaked on XLT4 agar plates and incubated at 37°C for 24 h. Colonies exhibiting the characteristic *Salmonella* reaction on the plates were streaked on CHROMagar *Salmonella* (presumptive confirmation) and TSA plates and incubated at 37°C for 24 h. Five to 10 colonies typical of *Salmonella* were picked from the TSA plates and confirmed as *Salmonella* using specific antiserum.

3.6.4 Enrichment steps for *Listeria*

Listeria was detected using the method described in *Listeria* Chromogenic Agar ISO 11290:2004. A 25 g composite soil sample was mixed thoroughly with 225 mL of Demi Fraser broth and incubated at 30°C for 24 h. A 1 µL aliquot of the enriched sample was streaked on *Listeria* chromogenic agar and incubated at 37°C for 24 h. The formation of blue-green colonies on *Listeria* chromogenic agar confirmed the presence of *L. monocytogenes*.

3.6.5 Selection of rifampicin-resistant *Salmonella* serovars

Pure serotyped *Salmonella* serovars were streaked on TSA plates and incubated at 37°C for 24 h. Five single colonies from the TSA plate were used to inoculate 5 mL sterile phosphate buffer and mixed thoroughly using a vortex-mixer. To make a lawn culture, 100 µL of the mixture was spread on TSA plates and incubated at 37°C for 24 h. Sterile phosphate buffer (10 mL) was used to loosen the lawn culture from the TSA plates and the suspension was collected in a sterile tube to make a liquid inoculum. Antibiotic-resistant colonies were selected by iterative subculture on TSA that contained increasing concentrations of rifampicin (0.2 mL to 1.0 mL stock rifampicin and 0.4 g sodium pyruvate in 400 mL TSA). Rifampicin-resistant *Salmonella* was selected for all tested serovars and stability of all the serovars was checked. Glycerol (15% + TSB) stock cultures were prepared for each serovar as described in Chapter 3.5.2 and stored at -80°C. All TSA and XLD plates amended with rifampicin are referred to throughout this thesis as TSARP and XLDRP plates, respectively.

3.6.6 Experimental design and statistical analysis

A factorial design with three replications for each combination of treatments was used for the microcosm pot trial. Five factors (temperature, moisture, chicken manure, soil type and time) were tested. The microbial count data was log-transformed and a two-way interaction between the aforementioned factors was considered. A more detailed description of the experimental design and statistical analysis is given in Chapters 4.2.5 and 4.2.10, respectively.

A split-plot block design with the whole-plot treatment being cover crop (mustard, radish, sorghum and no cover crop) and the split plot treatment being solarisation or no solarisation, were used for the field experiment. As for the microcosm pot trial, the microbial count data was log-transformed. The split-plot, whole-plot and time of sampling (weekly) were considered as fixed effects, whereas, block was considered as a random effect in the model. A more detailed description of the design field experimental and statistical analysis is given in Chapters 5.2.5 and 5.2.16, respectively.

4. Microcosm pot trial: *Salmonella* survival under controlled conditions

4.1 Introduction

Salmonella is more persistent in soil compared to other vegetative bacterial pathogens (Guan and Holley 2003; Arthurson *et al.* 2011), displaying long periods of survival (Zibilske and Weaver 1978) and only a slight reduction in cell numbers over time (Guo *et al.* 2002a). *Salmonella* inoculated in moist soil at 8.0 $\log_{10}$ CFU g^{-1} showed less than 2.0 log reduction after 45 days of storage at 20°C (Guan and Holley 2003). Arthurson *et al.* (2011) reported the persistence of *Salmonella* in soil without showing a significant reduction in bacterial numbers over a 4-week period, and it has also been reported that *Salmonella* survived from a few days up to 332 days in manure-amended soils (Islam *et al.* 2004c; Arrus *et al.* 2006; Holley *et al.* 2006; You *et al.* 2006). Variation in length of the survival period is attributed to various factors such as physical and chemical properties of the soil, temperature, moisture and strain type (van Veen *et al.* 1997). Most studies have investigated soil characteristics independently in relation of *Salmonella* survival, however, it is more likely that it is the interaction of these characteristics along with the various environmental factors that affects the survival of *Salmonella* (Semenov 2008).

Soil properties that affect bacterial survival include pH, organic matter content, nutrient availability, moisture, texture and particle size distribution (Chandler and Craven 1980; Ongeng *et al.* 2015). The effect of pH on survival of *S. enterica* in an agricultural matrix such as soil can be estimated from the impact of pH on the physiological and metabolic activities of the bacteria (Ongeng *et al.* 2015). Nutrient availability is found to be an important factor in affecting the survival and re-growth of eneteric pathogens in the soil. Since the physical and chemical properties of the soil vary with different soil types, the survival of bacterial pathogens is also reported to vary based on the properties of different soil types. For example, studies on *Escherichia coli* reported that longer survival was associated with soil with relatively more clay particles compared to sand particles (Semenov 2008).

Contaminated soil may act as a vector and a source of important human disease agents. Many diseases associated with soils (e.g. *Aspergillus fumigatus*, *Clostridium botulinum*, *C. tetani*) have been described, however survival and behaviour of enteric pathogens in soil is a

relatively new area, and as a consequence, the extent of disease transmission has possibly been underestimated (Santamaria and Toranzos 2003).

In regard to temperature, *Salmonella* is considered to be mesophilic although some strains are able to survive at extremely low or high temperatures (2–54°C) (Andino and Hanning 2015). However, most *Salmonella* serotypes grow over the fairly wide temperature range 7–48°C (Lawley 2013). Semenov (2008) reported that temperature is a key factor on survival, having strong effects on the longevity of enteric pathogens in manure and soil.

Temperature has a profound effect on the population dynamics of culturable bacteria (Ongeng *et al.* 2015). It influences the movement, survival and passive diffusion of bacteria in the soil and bacterial adsorption to soil particles may also be affected which will consequently affect retention of microbes within the soil matrix (Kemp *et al.* 1992). Higher temperatures generally induce more activity in bacteria while survival is prolonged at lower temperatures. It is also likely that temperature affects the diversity and behavior of resident microbial communities in manure and soil matrices which in turn affect enteropathogen survival.

The other important factor affecting survival of enteric pathogens is the moisture status of the soil. For microorganisms to grow and survive, moisture is a primary physiological requirement (Holley *et al.* 2006; Ongeng *et al.* 2015). Low soil moisture can either reduce the survival rates of enteric bacteria or force them into a persister stage (Himathongkham and Riemann 1999), although quantitative information is lacking (Jamieson *et al.* 2002). Most of the previous research has focused on the survival of enteric pathogens in moist or dry soil but not in different regimes such as constant and fluctuating moisture levels likely to be encountered in the field. Lawley (2013) reported that *Salmonella* is not able to grow in dry environments and cells will die in soil with low moisture content (Andino and Hanning 2015). Many studies have reported on just the survival times of enteropathogens in manure or manure-amended soil, rather than the importance of other factors such as pH, moisture, soil classification and presence of nutrients which together may influence human enteropathogen survival (McClure and Hall 2000). There are very few studies which show the interaction of these various factors in naturally existing in soils on the survival of enteric bacterial pathogens (Guan and Holley 2003).

Microbial responses observed in field situations are subject to intricate multivariate factors associated with continuously changing environmental and soil conditions that are often

difficult to interpret. However, the effects of specific factors on soil enteric pathogens can be studied under controlled conditions in the laboratory (Opperman *et al.* 1989; Fenlon *et al.* 2000). For example, microcosm studies using a consistent, reproducible and contained system make it possible to study particular factors that affect the behaviour of bacterial pathogens (Kemp *et al.* 1992). Therefore, microcosms or other controlled studies using soil help us to understand how environmental factors affect survival and allow us to create approaches that minimise the risk of enteropathogen survival and distribution within the food supply chain, commencing on-farm.

Little research has been conducted in Australia to determine the effect of local soil and climatic conditions on the survival of *Salmonella* in the environment. Because it is difficult to analyse all of the possible abiotic and biotic factors affecting the survival of *Salmonella* in a single study, the major factors such as temperature, moisture and soil properties were considered under controlled laboratory conditions in a microcosm pot trial in an effort to interpret the survival pattern of *Salmonella*. The results obtained using this strategy will help to better predict the survival of *Salmonella* in natural substrates such as soil or manure-amended soil under field conditions (see Chapter 5).

4.2 Materials and methods

4.2.1 Site description

Soil was obtained from the 0–10 cm layer from Karalee Farm and Pye Farm at the University of Sydney. Detailed descriptions of both research sites and soil collection are presented in Chapter 3.1.

4.2.2 Soil analysis

The chemical and physical properties of the soil were analysed and as described in Chapter 3.2.2.

4.2.3 Baseline characterisation of soil and chicken manure samples

Pure chicken manure (CM), which had been aged in large piles for up to 6 months, was obtained from a commercial source (Enfield Produce, Pet and Garden Supplies, Sydney, Australia) and used as a soil amendment for the microcosm pot trial. The presence/absence of *Salmonella* in the chicken manure or soil was tested by the following method before the start

of the trial. Replicate samples (100 g) of CM or soil (n = 3) were weighed and transferred to 400 mL sterile stomacher bags (Interscience, France). Aliquots of 150 mL of buffered peptone water (BPW) (Difco, USA) was added to each bag and incubated for 24 h at 37°C. This was followed by a second enrichment step (described in Chapter 3.6.3). The soil and CM samples used for sample preparation were either negative or below the limit of detection (<1 CFU g^{-1} CM) for *Salmonella*.

4.2.4 Preparation of soil and chicken manure samples

Soil samples (approximately 100 kg each of clay and sandy soils) were taken from several locations (n = 3) at the farms described above, sieved (2 mm mesh) and mixed thoroughly by hand to produce a composite sample. Soil only (100 g) or 98 g soil + 2 g CM for each soil was weighed into 120 mL plastic screw-cap pots (Sarstedt, Australia) ready for inoculation. The aliquot of chicken manure was mixed into each soil sample by shaking and stirring with a thin spatula.

4.2.5 Treatment and experimental design

The microcosm pot trial was a factorial design with three replications and five factors. The five factors were temperature, moisture, chicken manure, soil type and time. Levels of the different factors were (i) 5, 21 and 37°C, (ii) constant and fluctuating moisture, (iii) with and without manure, (iv) clay soil and sandy soil, and (v) incubation times of 1, 8, 15, 22, 29, 36 and 43 days. *Salmonella* serovars used for this trial were *S.* Enteritidis, *S.* Montevideo, *S.* Sofia and a cocktail of serovars including *S.* Enteritidis, *S.* Infantis, *S.* Montevideo, *S.* Typhimurium and *S.* Zanzibar. Control treatments consisted of 98 g soil + 2 g CM and 100 g soil only without addition of bacteria. Table 4.1 shows the arrangement of treatments. There were 2016 treatments in total.

Table 4.1 Arrangement of treatment factors and levels for the microcosm pot trial. The cocktail was comprised of *S*. Enteritidis, *S*. Infantis, *S*. Montevideo, *S*. Typhimurium and *S*. Zanzibar.

Factor	Levels
Inoculum	*S*. Enteritidis, *S*. Montevideo, *S*. Sofia, *Salmonella* cocktail
Soil type	Clay loam ('clay') coarse sand ('sandy')
Manure amendment	0%, 2% (w/w) poultry manure in soil
Incubation temperature	5, 21, 37°C
Moisture	Constant, fluctuating

4.2.6 Inoculum preparation and inoculation

S. enterica serovars Enteritidis, Infantis, Montevideo, Sofia, Typhimurium and Zanzibar were used in the microcosm pot trial (see Chapter 3.5.1 for source of serovars) and the identity of the serovars was checked using *Salmonella* antisera (see Chapter 3.4.1). From serotyped pure cultures, five colonies were taken into 5 mL phosphate buffer (Difco, USA) and mixed by vortexing. Each serovar was spread (100 µL) onto TSA plates (Difco, USA) and incubated for 24 h at 37°C to make a lawn culture (for lawn culture preparation see Chapter 3.6.5). A liquid inoculum (approximately 2.5 L for each serovar from 10 plates) was prepared by rinsing the lawn culture from TSA plates with phosphate buffer. The method for producing lawn cultures is provided in Chapter 3.6.5.

An inoculum cocktail was prepared by combining serovars Enteritidis, Infantis, Montevideo, Typhimurium and Zanzibar in equal proportions. A liquid inoculum cocktail was prepared in the same way as described above for individual serovars and equal proportions of the five cultures were adjusted using spectrophotometry at 600 nm. Optical adjustment was also done for the individual serovars. To determine a more accurate concentration of *Salmonella* cells in each inoculum (cocktail or individual inoculum), an aliquot from each inoculum was plated on XLD agar, incubated for 24 h at 37°C and enumerated. Optical density (OD) from 0.2–0.8 corresponded with population in the order of magnitude of 10^8 CFU mL^{-1}. The adjusted viable count for each serovar is presented in Table 4.2 and the proportion of each *Salmonella* serovars used to prepare cocktail inoculum is presented in Table 4.3. The inoculum level was selected to ensure sufficient changes in counts were measured over the life of the trial.

An aliquot (5 mL) of liquid suspension of either individual or cocktail cultures was added to each of the relevant microcosm pots (3 replications per treatment, 504 pots per serovar, 2016 samples in total for four inocula). For logistical and time management reasons, the starting time of incubation was staggered. Microcosm pots inoculated with *S.* Montevideo and *S.* Sofia were started first and pots inoculated with *S.* Enteritidis and the cocktail were initiated later. An additional 5 mL sterile water was added to each soil sample to achieve an initial moisture content of 15% (w/w) for the sandy soil and 20% (w/w) for the clay soil. The pots were labelled from 1–2016 (as pot ID). Control treatments (no inoculum, 100 g soil only or 98 g soil + 2 g CM) were inoculated with phosphate buffer (without inoculum) and treated in the same way as other treatments.

To get good dispersion of soil and liquid inoculum, the pots were capped and mixed thoroughly using a drum-mixer (Baldor, USA) for 20 min. After mixing, the lids remained in place for the constant moisture treatments and lids were removed from the tubes for fluctuating moisture treatments and covered with cheese-cloth fastened securely with a rubber band. Twenty four pots of all treatments in three replications were placed in a 5 L plastic box during the incubation period. For the microcosm pots in the fluctuating moisture treatments, the containment box was also covered with cheese-cloth secured with tape. All the pots in plastic boxes were kept in their respective temperature-controlled chambers (5, 21, 37°C).

4.2.7 Incubation conditions

The 120 mL plastic soil pot including the entire contents was weighed and the mass recorded on the container. Changes in moisture content was monitored by periodically selecting and weighing random samples. Weight loss from the content of pots was used to maintain the preset moisture content (15% [w/w] and 20% [w/w] for sandy and clay soils respectively). When the mass of fluctuating moisture samples decreased by 10 g, the moisture content was replenished was by adding 10 mL sterile water. Sterile water was added in a similar way to the constant moisture tubes to maintain constant moisture levels throughout the incubation period. The frequency of water addition was dependent on the storage temperature with more water added to microcosm pots held at high temperature (37°C) than low temperature (5°C) (Figure 4.1). During the incubation period, temperature was monitored using a minimum-maximum thermometer positioned inside each incubator.

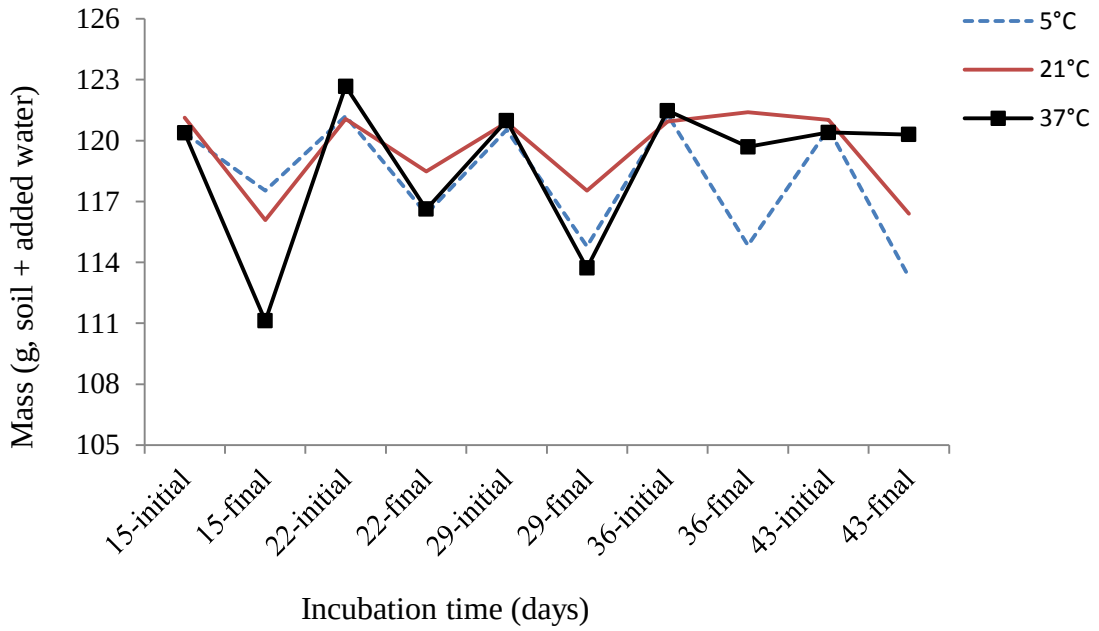


Figure 4.1 The extent of variation in soil moisture (g) in the fluctuating moisture regime at different temperatures (5, 21 and 37°C) over 43 days of incubation period of the mesocosm pot trial.

4.2.8 Sampling and enumeration

For destructive sampling, the entire contents of each microcosm pot (100 g) was aseptically transferred to a sterile stomacher bag (Interscience, France). A 100 mL aliquot of extraction buffer (Na_3PO_4 + Tween 20) was added using a gravimetric dilutor (Interscience, France), resulting in 1:2 (w/v) solution. The mixture was then extracted and enumerated as for the soil samples described in Chapter 3.6.2.

During enumeration, all XLD plates were checked and sorted into three categories: (i) countable plates, (ii) plates with overlapping colonies or crowded plates, and (iii) plates with no growth (Figure 4.2). Plates judged to be countable were enumerated using an automatic colony counter (Interscience, France). The following day, re-plating was done for plates with overlapping colonies after serially diluting the corresponding sample that was kept at 5°C. Re-plating (without dilution) was conducted for samples that exhibited no growth after dilution. After enumeration (from randomly selected plates), 5–10 colonies typical of *Salmonella* were picked from XLD plates and streaked on TSA plates for confirmation using specific antiserum.

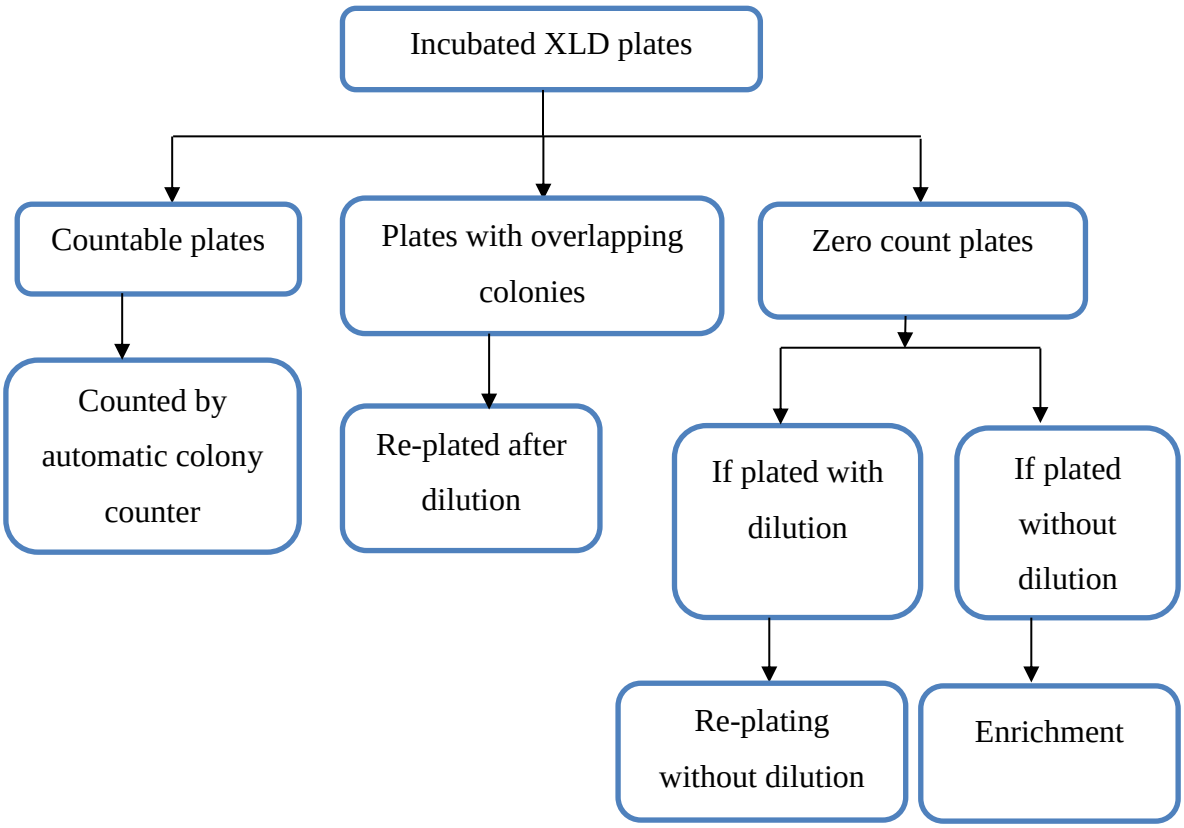


Figure 4.2 Schematic representation of the steps followed for plating and enrichment of *Salmonella* samples. XLD = Xylose lysine deoxycholate.

4.2.9 Enrichment steps for *Salmonella*

Samples from the zero-count plates (being spread without dilution) were enriched as described in Chapter 3.6.3 to check for the presence of viable *Salmonella* cells.

4.2.10 Statistical analysis

Salmonella count data was log-transformed and a mixed model in JMP Pro version 11 (SAS 2014, Cary, NC, USA) was used to analyse the data. Because of the large number of treatments, only two-way interactions between the factors temperature, moisture, soil, manure, *Salmonella* serovar and time were considered. This facilitated the analysis of the data and allowed inspection of two factors at a time. Data was pooled by combining all measurements from temperature, moisture, soil type and serovars for statistical analysis.

The factors were fitted as a fixed effect in the model whereas pot ID was fitted as a random effect. When there were significant interactions between factors, a post-hoc test was performed using Tukeys honest significant difference (HSD) to separate the means (an example of this method of analysis is given in Appendix 2). *Salmonella* presence/absence data was not statistically analysed since nearly all the results were found to be positive. In addition, in order to show the actual rate of *Salmonella* decline between treatments, an effect size was calculated. An effect size is the difference between two group means divided by the standard deviation of the two conditions (pooled standard deviation). Effect size measurements tell us the comparative magnitude of experimental treatments (Thalheimer and Cook 2002). Effect size was calculated using the following formula as described in Thalheimer and Cook (2002):

$d = (M_1 - M_2)/\delta$

d = Cohen's d effect size

M_1= mean of group one

M_2 = mean of group two

δ = pooled standard deviation.

When d = 0.2, 0.5, and 0.8 between two treatment groups, the effect size is considered to be small, medium and large respectively (Vasilopoulos *et al*. 2016).

4.3 Results

Prior to inoculation with an individual serovar or a cocktail of *Salmonella* serovars, chicken manure and soil were checked for the presence of naturally occurring *Salmonella*. Both the soil and chicken manure samples were found to be free from *Salmonella*.

The starting concentration of each *Salmonella* serovar or cocktail after enumeration is indicated in Table 4.2. The proportion of each *Salmonella* serovar in the cocktail inoculum is indicated in Table 4.3.

Table 4.2 Concentrations (log_{10} CFU g^{-1}) of *Salmonella* serovar liquid inoculum used in the microcosm pot trial. CFU = colony forming unit.

Inoculum	**Soil inoculation (log_{10} CFU g^{-1})**
Salmonella cocktail	5.1 ± 0.7
S. Enteritidis	5.1 ± 0.7
S. Montevideo	4.8 ± 0.1
S. Sofia	5.4 ± 0.6

Table 4.3 Concentrations (log_{10} CFU g^{-1}) of *Salmonella* serovars used to prepare the cocktail inoculum. CFU = colony forming unit.

Cocktail constituents	**Liquid inoculum (log_{10} CFU g^{-1})**
S. Enteritidis	8.2 ± 0.8
S. Infantis	8.4 ± 0.5
S. Montevideo	8.4 ± 0.7
S. Typhimurium	8.3 ± 0.8
S. Zanzibar	8.5 ± 0.7

4.3.1 Soil physicochemical properties

The soil from Pye Farm was described as clay loam (‘clay soil’) and soil from Karalee Farm was a coarse sand (‘sandy soil’) (Table 4.4). The total carbon and nitrogen content of the clay soil was approximately five and seven times greater, respectively, than the sandy soil. In addition, the amount of nitrate and ammonium in the clay soil was at least five times greater

than sandy soil. Other analyses including bulk density, soil moisture, pH and EC indicate the main differences between the two soil types (Table 4.4).

Table 4.4 Physicochemical properties of the two soil types from Karalee Farm and Pye Farm at the University of Sydney with and without chicken manure amendment. Values are mean ± standard deviation. C = carbon, N = nitrogen, EC = electrical conductivity, CM = chicken manure, µS = microSiemens, [1]sandy soil obtained from Karalee Farm, Camden, [2]clay soil obtained from Pye Farm, Bringelly.

Variables	**Sandy[1]**	**Sandy + CM**	**Clay[2]**	**Clay + CM**
Colour	Brown		Light grey	
Texture	Coarse sand		Clay loam	
Bulk density (g cm^{-3})	1.28 ± 0.09		1.12 ± 0.03	
Total N (%)	0.053 ± 0.003	0.111 ± 0.008	0.383 ± 0.003	0.436 ± 0.009
Total C (%)	0.722 ± 0.029	1.099 ± 0.083	4.242 ± 0.087	4.518 ± 0.080
Ammonium (mg kg^{-1})	1.23 ± 0.42	5.27 ± 0.30	7.66 ± 0.63	10.03 ± 1.14
Nitrate (mg kg^{-1})	2.54 ± 0.26	2.27 ± 0.37	15.04 ± 2.19	14.70 ± 3.34
Soil moisture (%)	0.08 ± 0.01	0.08 ± 0.01	0.15 ± 0.01	0.15 ± 0.02
pH	5.41 ± 0.07	7.53 ± 0.14	5.06 ± 0.04	6.19 ± 0.35
EC (µS)	85.38 ± 4.19	592.60 ± 74.64	512.40 ± 16.71	907.20 ± 189.61

4.3.2 Effect of temperature and time on survival of *Salmonella*

The highest incubation temperature (37°C) led to a more rapid decline in *Salmonella* numbers compared to both lower incubation temperatures (Figure 4.3). As a result, there was a significant (P <0.001) difference among the three temperature treatments affecting the decline of *Salmonell*a over the 43 day incubation period.

Throughout the incubation period, there were significant differences in *Salmonella* numbers between lower (5°C) and higher temperatures (37°C) except at day 1 and day 43. Over the incubation period, *Salmonella* counts were reduced on average by 1.30, 0.80 and 0.94 $\log_{10}$ CFU g^{-1} in samples stored at 37, 21 and 5°C, respectively. *Salmonella* counts for microcosm pots incubated at the intermediate temperature (21°C) were not significantly different from either the low temperature (5°C) or high temperature (37°C) throughout the incubation period, except at day 8 (Table 4.5). The effect size between intermediate (21°C) and low (5°C) temperatures and intermediated (21°C) and high (37°C) temperatures was found to be

low whereas, the effect size between low (5°C) and high (37°C) temperatures was found to be medium (data not shown).

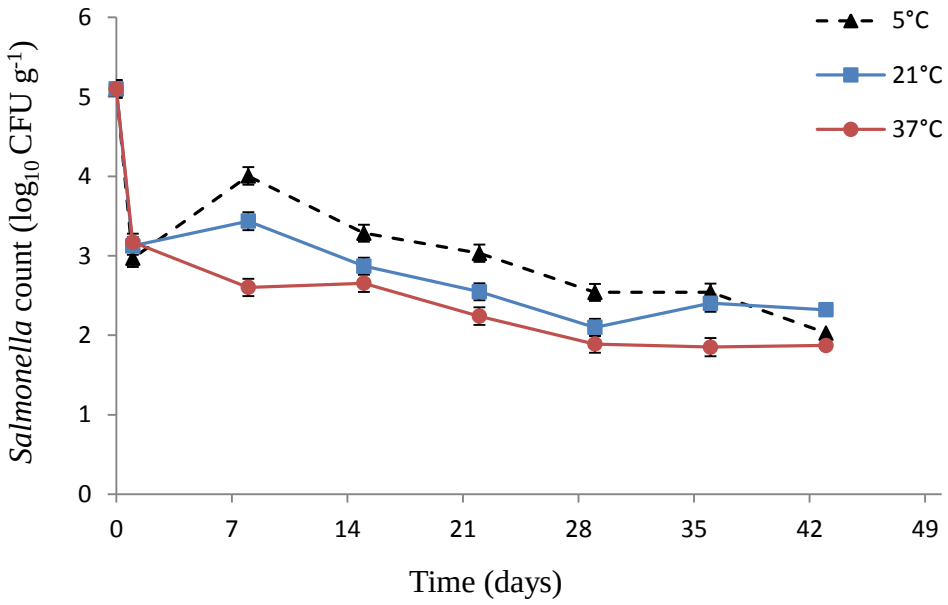


Figure 4.3 The effect of temperature (°C) and time (days) on the survival of *Salmonella* (log_{10} CFU g^{-1}). Data were pooled from four factors (moisture, soil type, presence/absence of manure and serovars). Data points are mean ± standard error, n = 96, *P* <0.001. The quantitative limit of detection (LOD; 1.18 log_{10} CFU g^{-1}) of the viable count was determined by direct plating. CFU = colony forming unit.

Irrespective of temperature, a rapid decline in *Salmonella* numbers of approximately 2.0 log_{10} CFU g^{-1} was measured 1 day after inoculation. At 5°C, a subsequent increase in numbers of approximately 1.0 log_{10} CFU g^{-1} occurred (day 7) and thereafter numbers declined linearly. In contrast, the response at 37°C was biphasic with the rapid reduction after inoculation followed by a linear decrease from 3.10 to 1.90 log_{10} CFU g^{-1} for the duration of the trial. At 21°C, an intermediate response was observed with a minor increase of 0.31 log_{10} CFU g^{-1} occurring 1 day after inoculation followed by a linear decrease.

Table 4.5 Effect of temperature (°C) and time (days) on *Salmonella* survival (log_{10} CFU g^{-1}). Values are mean ± standard error, n = 96. Data were pooled from four factors (moisture, soil type, presence/absence of manure and serovar) for statistical analysis. Mean values followed by the same letter are not significantly different (Tukeys HSD post-hoc test). CFU = colony forming unit.

	***Salmonella* count (log_{10} CFU g^{-1})**		
	Temperature (°C)		
Day	**5**	**21**	**37**
1	2.97 ± 0.11^{fghij}	3.12 ± 0.11^{ghij}	3.17 ± 0.11^{hij}
8	4.00 ± 0.11^{k}	3.44 ± 0.11^{j}	2.60 ± 0.11^{cdefg}
15	3.28 ± 0.11^{ij}	2.87 ± 0.11^{efghi}	2.66 ± 0.11^{defgh}
22	3.03 ± 0.11^{fghij}	2.55 ± 0.11^{bcdef}	2.24 ± 0.11^{abcd}
29	2.54 ± 0.11^{bcdef}	2.10 ± 0.11^{abc}	1.89 ± 0.11^{a}
36	2.54 ± 0.11^{bcdef}	2.40 ± 0.11^{abcde}	1.85 ± 0.11^{a}
43	2.03 ± 0.11^{ab}	2.32 ± 0.11^{abcde}	1.87 ± 0.11^{a}

4.3.3 Effect of soil type and time on survival of *Salmonella*

There was a significant difference between clay and sandy soils on the survival of *Salmonella.* Generally, levels of *Salmonella* decreased with incubation time however, the survival of *Salmonella* in sandy soil was less than that observed for clay soil (Figure 4.4). *Salmonella* survival was higher in clay soil compared with sandy soil at all times except day 8 which had similar *Salmonella* counts to those observed in clay soil at day 29 and day 43. *Salmonella* counts in clay soil at the end of the incubation period were the same as the counts recorded for sandy soil at the beginning of the incubation period (Table 4.6). In addition, a large effect size (d = 1.47) was observed between the two treatments (sandy and clay soils, data not shown).

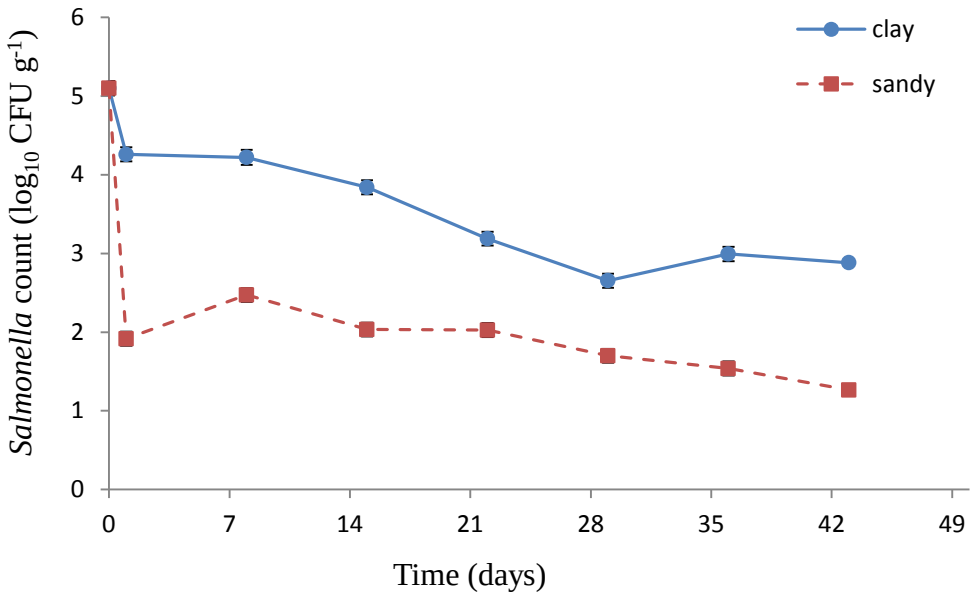


Figure 4.4 Effect of soil type and time (days) on survival of *Salmonella* (log_{10} CFU g^{-1}). Data were pooled from four factors (temperature, moisture, presence/absence of manure and serovar). Data points are mean ± standard error, n = 144, P <0.001. The quantitative limit of detection (LOD; 1.18 log_{10} CFU g^{-1}) of the viable count was determined by direct plating. CFU = colony forming unit.

Table 4.6 The effect of soil type and time (days) on survival of *Salmonella* (log_{10} CFU g^{-1}). Values are mean ± standard error, n = 144. Data were pooled from four factors (temperature, moisture, presence/absence of manure and serovar) for statistical analysis. Mean values followed by the same letter are not significantly different (Tukeys HSD post-hoc test). CFU = colony forming unit.

	***Salmonella* count (log_{10} CFU g^{-1})**	
Day	**Clay soil**	**Sandy soil**
1	4.26 ± 0.09^{g}	1.92 ± 0.09^{bc}
8	4.22 ± 0.09^{g}	2.47 ± 0.09^{d}
15	3.84 ± 0.09^{g}	2.03 ± 0.09^{c}
22	3.19 ± 0.09^{f}	2.02 ± 0.09^{c}
29	2.65 ± 0.09^{de}	1.70 ± 0.09^{bc}
36	2.99 ± 0.09^{ef}	1.54 ± 0.09^{ab}
43	2.88 ± 0.09^{def}	1.26 ± 0.09^{a}

4.3.4 Effect of manure amendments and time on survival of *Salmonella*

The effect of manure amendments on *Salmonella* survival was found to be significant (*P* <0.0001) over the 43 day incubation period. *Salmonella* survival decreased over time both with and without manure amendments. However, the decline in *Salmonella* counts was found to be at least 1.0 $\log_{10}$ CFU g^{-1} greater in the absence of manure when compared to counts observed in samples with manure amendments (Figure 4.5). Over the incubation period, the average decline of *Salmonella* was 0.69 and 1.33 $\log_{10}$ CFU g^{-1} in no-manure and manure-treated samples, respectively. In addition, in order to show the actual rate of decline, effect size was calculated and large d-value (0.99) was observed between the two treatments (with and without manure amendments) (data not shown).

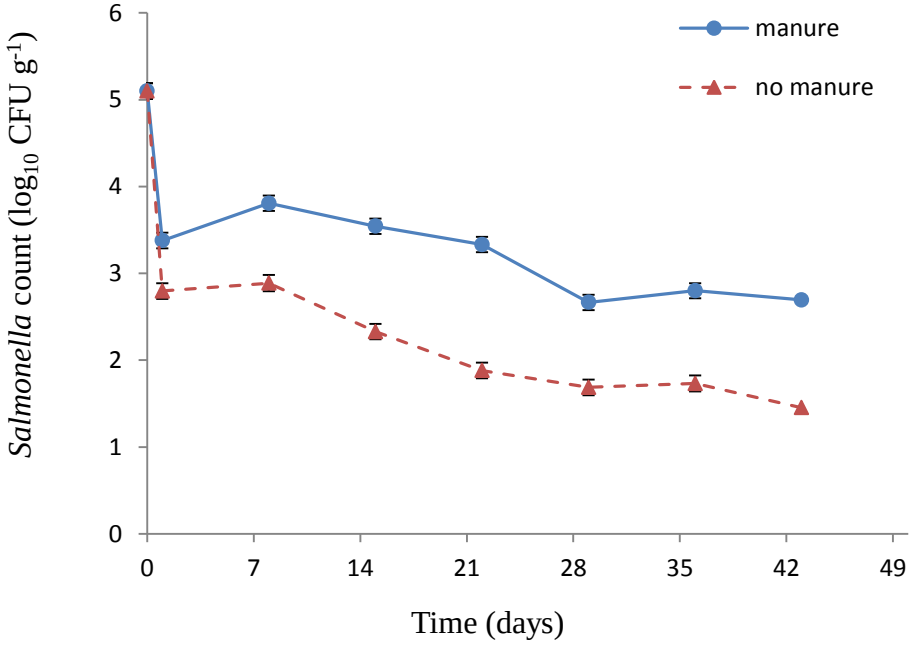


Figure 4.5 Effect of manure amendments and time (days) on survival of *Salmonella* ($\log_{10}$ CFU g^{-1}). Data were pooled from four factors (temperature, moisture, soil type and serovars). Data points are mean ± standard error, n = 144, *P* <0.001. The quantitative limit of detection (LOD; 1.18 $\log_{10}$ CFU g^{-1}) of the viable count was determined by direct plating. CFU = colony forming unit.

Salmonella counts were reduced on average by 20% in manure treated samples and 48% in samples without manure over the incubation period (Table 4.7).

Table 4.7 The effect of manure amendment and time (days) on survival of *Salmonella* (log_{10} CFU g^{-1}). Values are mean ± standard error, n = 144. Data were pooled from four factors (temperature, moisture, soil type and serovar) for statistical analysis. Mean values followed by the same letter are not significantly different (Tukeys HSD post-hoc test). CFU = colony forming unit.

	***Salmonella* count (log_{10} CFU g^{-1})**	
Day	**With manure**	**No manure**
1	3.38 ± 0.09^{d}	2.80 ± 0.09^{c}
8	3.80 ± 0.09^{e}	2.89 ± 0.09^{c}
15	3.54 ± 0.09^{de}	2.33 ± 0.09^{b}
22	3.33 ± 0.09^{d}	1.88 ± 0.09^{a}
29	2.66 ± 0.09^{bc}	1.69 ± 0.09^{a}
36	2.80 ± 0.09^{c}	1.73 ± 0.09^{a}
43	2.69 ± 0.09^{bc}	1.45 ± 0.09^{a}

4.3.5 Survival of *Salmonella* serovars over time

The interaction effect between time and serovars was significant ($P = 0.0226$) and counts for all the *Salmonella* serovars decreased with incubation time (Figure 4.6). The rate of decline for each serovar was similar from day 29 until the end of the incubation period (Table 4.8).

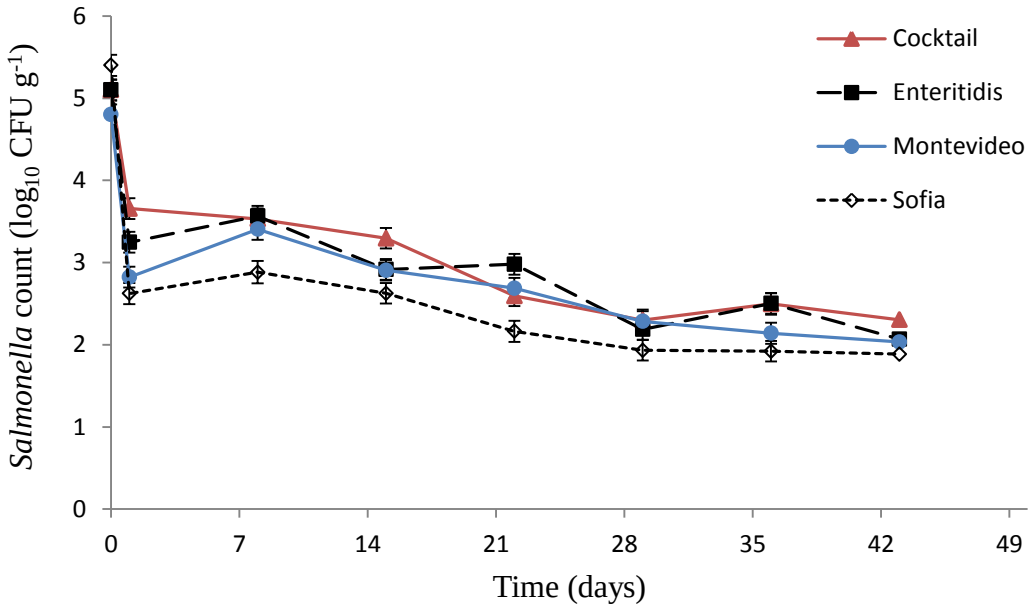


Figure 4.6 The effect of time (days) on survival of *Salmonella* serovars (log_{10} CFU g^{-1}). Data were pooled from four factors (temperature, moisture, soil type and presence/absence of manure). Data points are mean ± standard error, n = 72, P <0.0226. The quantitative limit of detection (LOD; 1.18 log_{10} CFU g^{-1}) of the viable count was determined by direct plating. CFU = colony forming unit.

Table 4.8 The effect of time (days) on survival of *Salmonella* serovars (log_{10} CFU g^{-1}). Data were pooled from four factors (temperature, moisture, soil type, and presence/absence of manure) for statistical analysis. Mean values followed by the same letter are not significantly different (Tukeys HSD post-hoc test). CFU = colony forming unit.

	***Salmonella* count (log_{10} CFU g^{-1})**			
Day	**Cocktail**	***S.* Enteritidis**	***S.* Montevideo**	***S.* Sofia**
1	3.66 ± 0.13^{l}	3.25 ± 0.12^{ghijkl}	$2.82 \pm 0.12d^{efghi}$	2.62 ± 0.13^{cdefg}
8	3.53 ± 0.12^{jkl}	3.57 ± 0.12^{kl}	3.41 ± 0.12^{ijkl}	2.88 ± 0.13^{efghij}
15	3.30 ± 0.12^{hijkl}	2.92 ± 0.12^{efghijk}	2.91 ± 0.13^{efghijk}	2.63 ± 0.14^{cdefgh}
22	2.59 ± 0.12^{bcdefg}	2.98 ± 0.13^{fghijk}	2.69 ± 0.12^{cdefgh}	2.16 ± 0.12^{abcd}
29	2.30 ± 0.12^{abcde}	2.19 ± 0.13^{abcd}	2.28 ± 0.12^{abcde}	1.93 ± 0.13^{ab}
36	2.50 ± 0.13^{abcdef}	2.50 ± 0.13^{abcdef}	2.14 ± 0.12^{abc}	1.92 ± 0.12^{a}
43	2.30 ± 0.13^{abcde}	2.07 ± 0.13^{abc}	2.03 ± 0.13^{abc}	1.89 ± 0.12^{a}

4.3.6 Effect of moisture and time on survival of *Salmonella*

Moisture had a significant effect (P <0.001) on the survival of *Salmonella* over time. After inoculation, the rate of decline looked similar for both treatments, albeit at counts of about 1.0 $\log_{10}$ CFU g^{-1} lower for fluctuating moisture compared to constant moisture. In addition to statistical tests of significance, effect size was calculated and large effect size (d = 0.81) was observed between the two moisture regimes (data not shown). Since the same amount of inoculum was used for both constant and fluctuating moisture, the difference in one order of magnitude between the two moisture regimes (Figure 4.7, Table 4.9) might have been due to the death of considerably more *Salmonella* cells in the fluctuating moisture treatments at the beginning of the experiment.

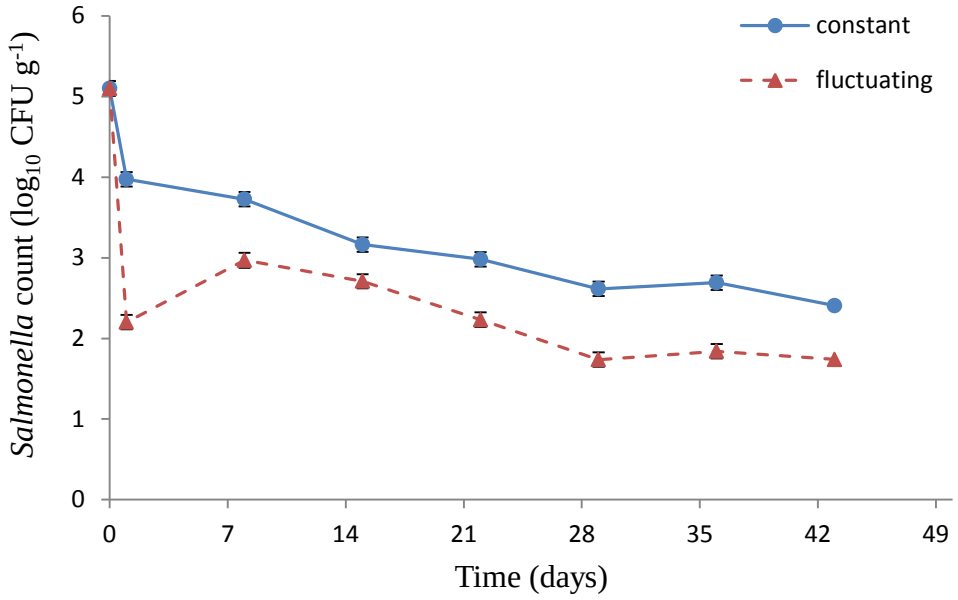


Figure 4.7 The effect of soil moisture and time (days) on survival of *Salmonella* ($\log_{10}$ CFU g^{-1}). Data were pooled from four factors (temperature, soil type, presence/absence of manure and serovar). Data points are mean ± standard error, n = 144, P <0.001. The quantitative limit of detection (LOD; 1.18 $\log_{10}$ CFU g^{-1}) of the viable count was determined by direct plating. CFU = colony forming unit.

Table 4.9 The effect of soil moisture and time (days) on survival of *Salmonella* (log_{10} CFU g^{-1}). Values are mean ± standard error, n = 144. Data were pooled from four factors (temperature, soil type, presence/absence of manure and serovar) for statistical analysis. Mean values followed by the same letter are not significantly different (Tukeys HSD post-hoc test). CFU = colony forming unit.

	***Salmonella* count (log_{10} CFU g^{-1})**	
Day	**Constant moisture**	**Fluctuating moisture**
1	3.97 ± 0.09^{g}	2.20 ± 0.09^{bc}
8	3.72 ± 0.09^{g}	2.97 ± 0.09^{ef}
15	3.16 ± 0.09^{f}	2.71 ± 0.09^{de}
22	2.98 ± 0.09^{ef}	2.23 ± 0.09^{bc}
29	2.61 ± 0.09^{cde}	1.74 ± 0.09^{a}
36	2.69 ± 0.09^{de}	1.84 ± 0.09^{ab}
43	2.41 ± 0.09^{cd}	1.74 ± 0.09^{a}

4.3.7 Effect of temperature and soil type on survival of *Salmonella*

The interaction of temperature and soil had a significant effect on the survival of *Salmonella.* Survival was greater in clay soil compared with survival in sandy soil at all temperatures (5, 21, 37°C) (Figure 4.8). The survival of *Salmonella* in sandy soil was not affected by the three temperature treatments; however the interaction effect of temperature with soil had a significant effect on the survival of *Salmonella* in clay soil (Table 4.10).

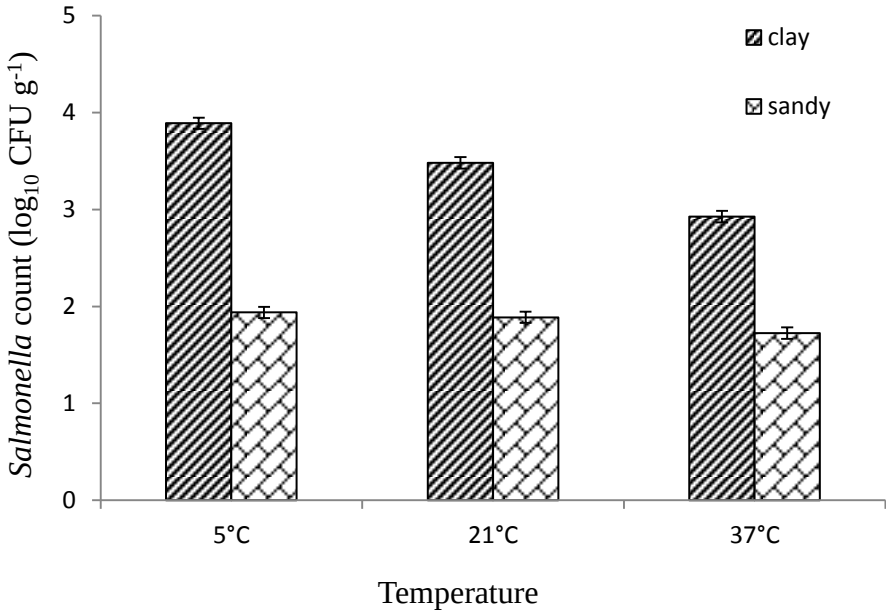


Figure 4.8 The survival of *Salmonella* (log_{10} CFU g^{-1}) at the conclusion of the microcosm pot trial in two soil types (clay and sandy) at three different temperatures (5, 21 and 37ºC). Data were pooled from four factors (moisture, presence/absence of manure, serovar and sampling time). Bars are mean ± standard error, n = 336, P <0.001. The quantitative limit of detection (LOD; 1.18 log_{10} CFU g^{-1}) of the viable count was determined by direct plating. CFU = colony forming unit.

Table 4.10 The effect of temperature (°C) and soil type on the survival of *Salmonella* (log_{10} CFU g^{-1}). Values are mean ± standard error, n = 336. Data were pooled from four factors (moisture, presence/absence manure, serovar, and sampling time) for statistical analysis. Mean values followed by the same letter are not significantly different (Tukeys HSD post-hoc test). CFU = colony forming unit.

	***Salmonella* count (log_{10} CFU g^{-1})**	
Temperature (°C)	**Clay soil**	**Sandy soil**
5	3.89 ± 0.06^{d}	1.94 ± 0.06^{a}
21	3.48 ± 0.06^{c}	1.89 ± 0.06^{a}
37	2.93 ± 0.06^{b}	1.72 ± 0.06^{a}

4.3.8 Effect of temperature and manure amendments on survival of *Salmonella*

The interaction between temperature and manure amendments had a significant effect on the survival of *Salmonella.* The addition of chicken manure significantly improved the survival of *Salmonella* across all temperatures when compared with samples incubated in soil without chicken manure amendments (Figure 4.9).

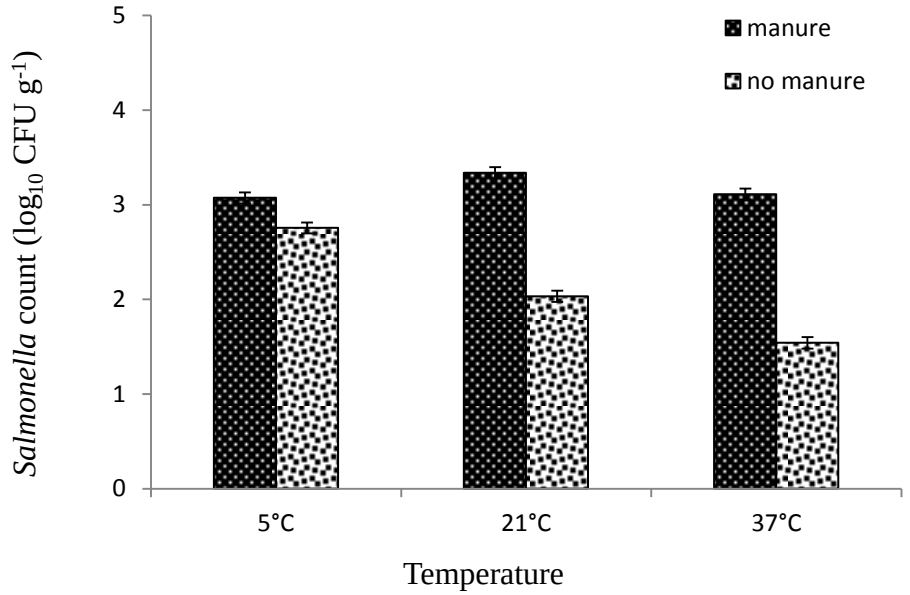


Figure 4.9 The effect of temperature (°C) and manure amendments on the survival of *Salmonella* (log_{10} CFU g^{-1}) at the conclusion of the microcosm pot trial. Data were pooled from four factors (moisture, soil type, serovar and sampling time). Bars represent mean ± standard error, n = 336, P <0.001. The quantitative limit of detection (LOD; 1.18 log_{10} CFU g^{-1}) of the viable count was determined by direct plating. CFU = colony forming unit.

In the presence of chicken manure, *Salmonella* survival was approximately 1.0 log_{10} CFU g^{-1} greater at the intermediate (21°C) and high (37°C) temperatures compared to the lower temperature (5°C). Similarly, when there was no manure amendment, *Salmonella* survival was about 1.0 log_{10} CFU g^{-1} greater at the lowest temperature (5°C) compared to the highest temperature (37°C) (Table 4.11).

Table 4.11 The effect of temperature (°C) and manure amendment on *Salmonella* survival (log_{10} CFU g^{-1}). Values are mean ± standard error, n = 336. Data were pooled from four factors (moisture, soil type, serovar and sampling time) for statistical analysis. Mean values followed by the same letter are not significantly different (Tukeys HSD post-hoc test). CFU = colony forming unit.

***Salmonella* count (log_{10} CFU g^{-1})**		
Temperature (°C)	**Manure amendment**	
	With manure	**No manure**
5	3.07 ± 0.06^{d}	2.76 ± 0.06^{c}
21	3.34 ± 0.06^{e}	2.03 ± 0.06^{b}
37	3.11 ± 0.06^{de}	1.54 ± 0.06a

4.3.9 Effect of temperature on survival of *Salmonella* serovars

The survival of *Salmonella* cocktail, *S*. Enteritidis and *S*. Montevideo was better at the lowest temperature (5°C) compared to the highest temperature (37°C) (Figure 4.10). There was no significant decline of *S*. Sofia in any of the temperature treatments (Table 4.12). *S*. Sofia was found to be the poorest survivor (Tables 4.12, 4.13) when compared with the other serovars. At 5°C, the survival was 40, 58, 64 and 67% for *S*. Sofia, *S*. Montevideo, *S*. Enteritidis and cocktail, respectively.

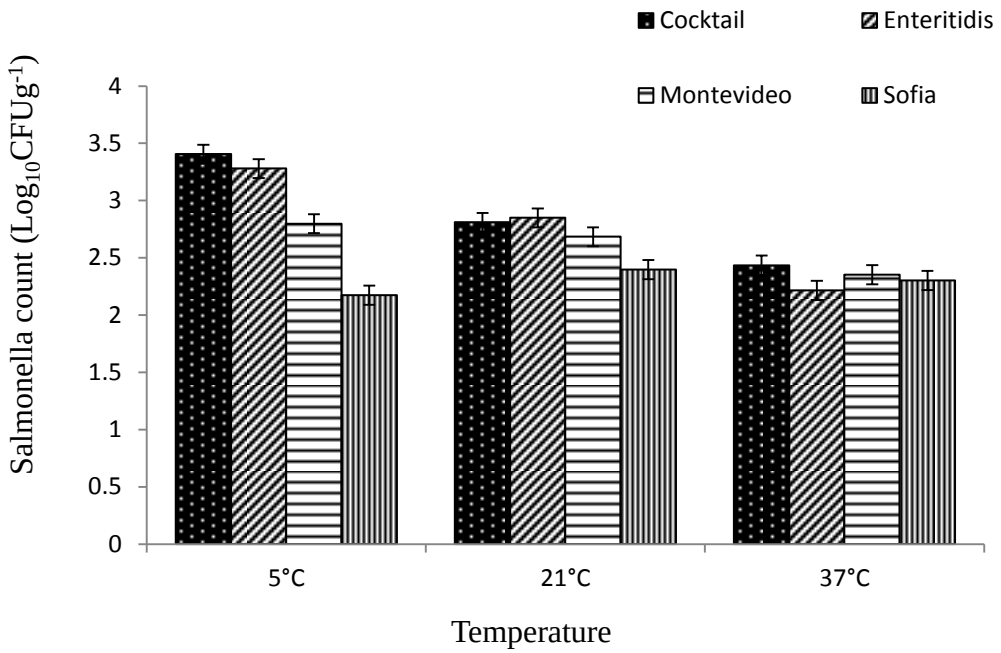


Figure 4.10 The effect of temperature (°C) on the survival of *Salmonella* serovars (log_{10} CFU g^{-1}). Data were pooled from four factors (moisture, soil type, presence/absence of manure and sampling time). Bars are mean ± standard error, n = 168, P <0.001. The quantitative limit of detection (LOD; 1.18 log_{10} CFU g^{-1}) of the viable count was determined by direct plating. CFU = colony forming unit.

Table 4.12 The effect of temperature (°C) on survival of different *Salmonella* serovars (log_{10} CFU g^{-1}). Values are mean ± standard error, n = 168. Data were pooled from four factors (moisture, soil type, presence/absence of manure and sampling time) for statistical analysis. Mean values followed by the same letter are not significantly different (Tukeys HSD post-hoc test). CFU = colony forming unit.

	***Salmonella* count (log_{10} CFU g^{-1})**			
Temperature (°C)	**Cocktail**	***S*. Enteritidis**	***S*. Montevideo**	***S*. Sofia**
5	3.40 ± 0.08^{e}	3.28 ± 0.08^{e}	2.80 ± 0.08^{cd}	2.17 ± 0.08^{a}
21	2.81 ± 0.08^{cd}	2.85 ± 0.08^{d}	2.68 ± 0.08^{bcd}	2.40 ± 0.08^{ab}
37	2.43 ± 0.09^{abc}	2.22 ± 0.08^{a}	2.35 ± 0.08^{ab}	2.30 ± 0.08^{ab}

Table 4.13 Proportional survival (%) of *Salmonella* serovars at different temperatures.

	***Salmonella* survival (%)**			
Temperature (°C)	**Cocktail**	***S*. Enteritidis**	***S*. Montevideo**	***S*. Sofia**
5	67	64	58	40
21	55	56	56	44
37	48	43	49	43

4.3.10 Effect of temperature and moisture interactions on survival of *Salmonella*

The interaction of moisture and temperature had a significant effect (P <0.001) on the survival of *Salmonella.* A lower incubation temperature and constant moisture favoured *Salmonella* survival (Figure 4.11). *Salmonella* counts reduced on average by 2.30, 1.08 and 1.42 $\log_{10}$ CFU g^{-1} in samples stored at 37, 21 and 5°C, respectively, under conditions of fluctuating moisture. In the constant moisture samples, the average reduction of *Salmonella* was 0.38, 0.51 and 0.49 $\log_{10}$ CFU g^{-1} in samples stored at 37, 21 and 5°C, respectively.

At the end of the incubation period, the number of *Salmonella* cells was found to be 2.61 $\log_{10}$ CFU g^{-1} at 37°C with constant moisture. This was not statistically different from the fluctuating samples incubated at 5°C, which had 2.39 $\log_{10}$ CFU g^{-1} soil (Table 4.14). The lowest number of *Salmonella* cells was in samples incubated at higher temperatures (21 or 37°C) under fluctuating moisture regimes.

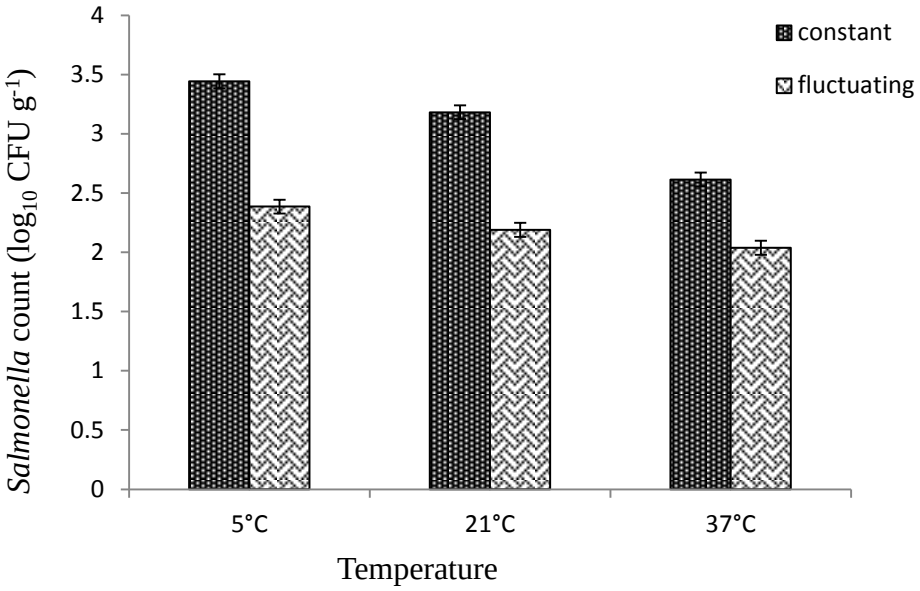


Figure 4.11 The effect of temperature (°C) and soil moisture on survival of *Salmonella* (log_{10} CFU g^{-1}). Data were pooled from four factors (soil type, presence/absence of manure, serovar and sampling time). Bars are mean ± standard error, n = 336, P <0.001. The quantitative limit of detection (LOD; 1.18 log_{10} CFU g^{-1}) of the viable count was determined by direct plating. CFU = colony forming unit.

Table 4.14 The effect of temperature (°C) and soil moisture on the survival of *Salmonella* (log_{10} CFU g^{-1}). Values are mean ± standard error, n = 336. Data were pooled from four factors (soil type, presence/absence of manure, serovar and sampling time) for statistical analysis. Mean values followed by the same letter are not significantly different (Tukeys HSD post-hoc test). CFU = colony forming unit.

Temperature (°C)	***Salmonella* count (log_{10} CFU g^{-1})**	
	Moisture	
	Constant	**Fluctuating**
5	3.44 ± 0.06^{e}	2.39 ± 0.06^{bc}
21	3.18 ± 0.06^{d}	2.19 ± 0.06^{ab}
37	2.61 ± 0.06^{c}	2.04 ± 0.06^{a}

4.3.11 Effect of soil type and manure amendments on survival of *Salmonella*

The interaction between soil types and manure amendments significantly affected the survival of *Salmonella.* Survival was found to be better in samples with manure amendments. The lowest *Salmonella* count was detected in sandy soil without manure amendments (Figure 4.12). In soils without added manure, *Salmonella* survival was greater in clay soil than sandy soil.

4.3.12 Effect of soil type and moisture interactions on survival of *Salmonella*

The interaction effect of moisture and soil significantly affected the survival of *Salmonella.* Survival was found to be higher in constant moisture conditions followed by fluctuating moisture in clay soil. In both soils, constant moisture appeared to favour *Salmonella* survival with the lowest cell count being detected in sandy soil with fluctuating moisture (Figure 4.13).

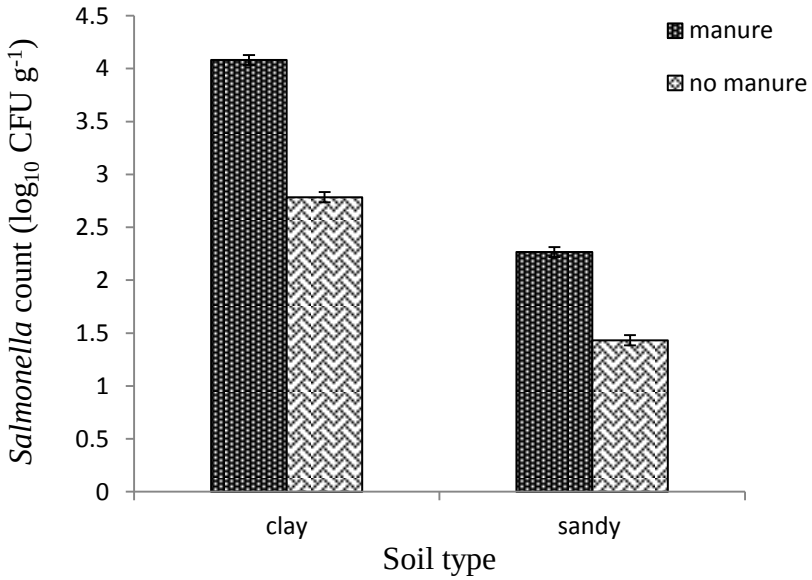


Figure 4.12 The effect of soil type and manure amendment interactions on the survival of *Salmonella* (log_{10} CFU g^{-1}). Data were pooled from four factors (temperature, moisture, serovar and sampling time). Bars are mean ± standard error, n = 504, P <0.001. The quantitative limit of detection (LOD; 1.18 log_{10} CFU g^{-1}) of the viable count was determined by direct plating. CFU = colony forming unit.

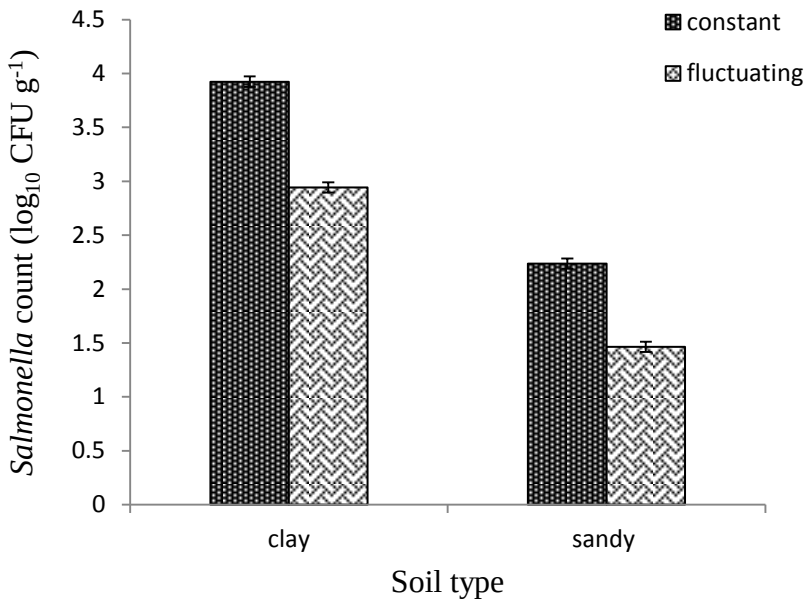


Figure 4.13 The effect of soil type and moisture regime on the survival of *Salmonella* (log_{10} CFU g^{-1}). Data were pooled from four factors (temperature, presence/absence of manure, serovar and sampling time). Bars are mean ± standard error, n = 504, P = 0.0285. The quantitative limit of detection (LOD; 1.18 log_{10} CFU g^{-1}) of the viable count was determined by direct plating. CFU = colony forming unit.

4.3.13 Effect of manure amendments and moisture regime on survival of *Salmonella*

The interaction effect between moisture and manure amendments significantly affected the survival of *Salmonella.* Survival was improved more in constant moisture conditions compared to fluctuating moisture in the presence of manure. The lowest *Salmonella* count was detected in unamended samples with fluctuating moisture (Figure 4.14). Some of the interaction effects were not significant such as soil with serovar (P = 0.444), manure with serovar (P = 0.250) and moisture with serovar (P = 0.686) (Appendix 2).

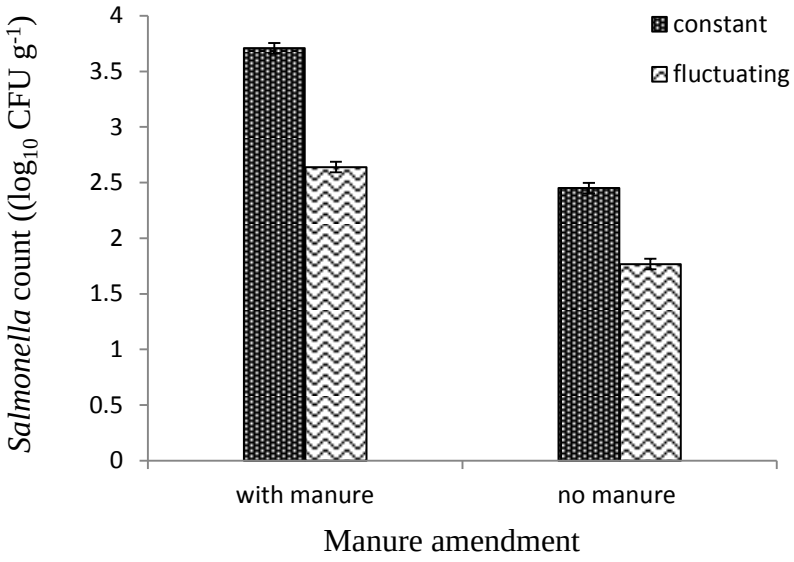


Figure 4.14 The effect of manure amendments and moisture regimes on the survival of *Salmonella* ($\log_{10}$ CFU g^{-1}). Data were pooled from four factors (temperature, soil type, serovar and sampling time). Bars are mean ± standard error, n = 504, P <0.001. The quantitative limit of detection (LOD; 1.18 $\log_{10}$ CFU g^{-1}) of the viable count was determined by direct plating. CFU = colony forming unit.

4.3.14 Presence/absence of *Salmonella* cells after enrichment

When the detection of *Salmonella* cells was found to be below the limit of detection (LOD; 1.18 $\log_{10}$ CFU g^{-1}) after direct counting, the corresponding samples were enriched to check the presence/absence of viable *Salmonella* cells. This was done by streaking samples on XLT4 agar (Figure 4.15a) and on *Salmonella* CHROMagar (Figure 4.15b) and confirmed using specific antiserum from randomly selected typical *Salmonella* colonies. Over 43 days of incubation, from the total number of samples (504 per serovar), 242, 200, 196 and 194 samples were enriched from *S.* Sofia, *S.* Montevideo, *S.* Enteritidis and the serovar cocktail inoculated soil samples, respectively.

The data obtained from enrichment (positive or negative) was not analysed because most of the enriched samples were found to be positive and, for the analysis of direct counts, enrichment data was considered in the overall model as a zero count. The proportion of direct count and positive or negative *Salmonella* cells for *Salmonella* cocktail is indicated in Figure 4.16 and represents a similar for the other serovars. The proportion of enriched samples was

greater in sandy soil (Figure 4.16b) than in sandy soil with manure amendments (Figure 4.16a). Similarly, the proportion of enriched samples was greater in clay soil (Figure 4.16d) than in clay with manure amendments (Figure 4.16c).

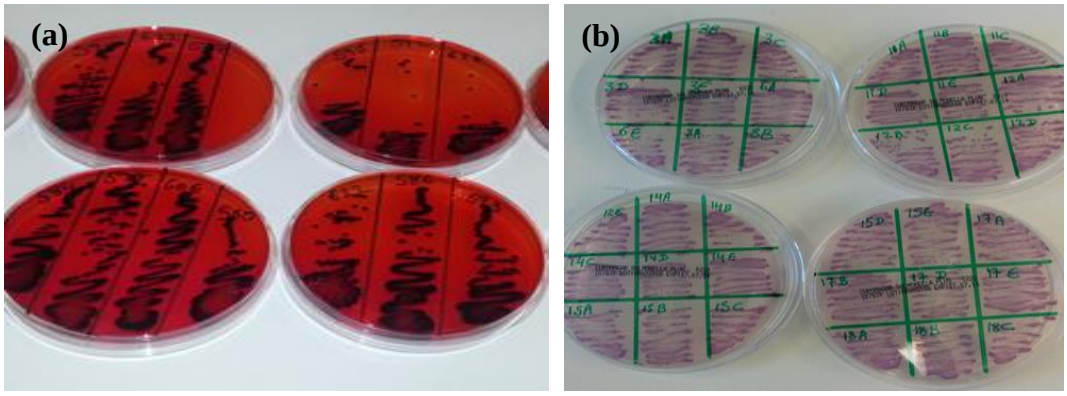


Figure 4.15 Black and mauve-coloured colonies formed by *Salmonella* on (a) XLT4 agar and (b) *Salmonella* CHROMagar, respectively.

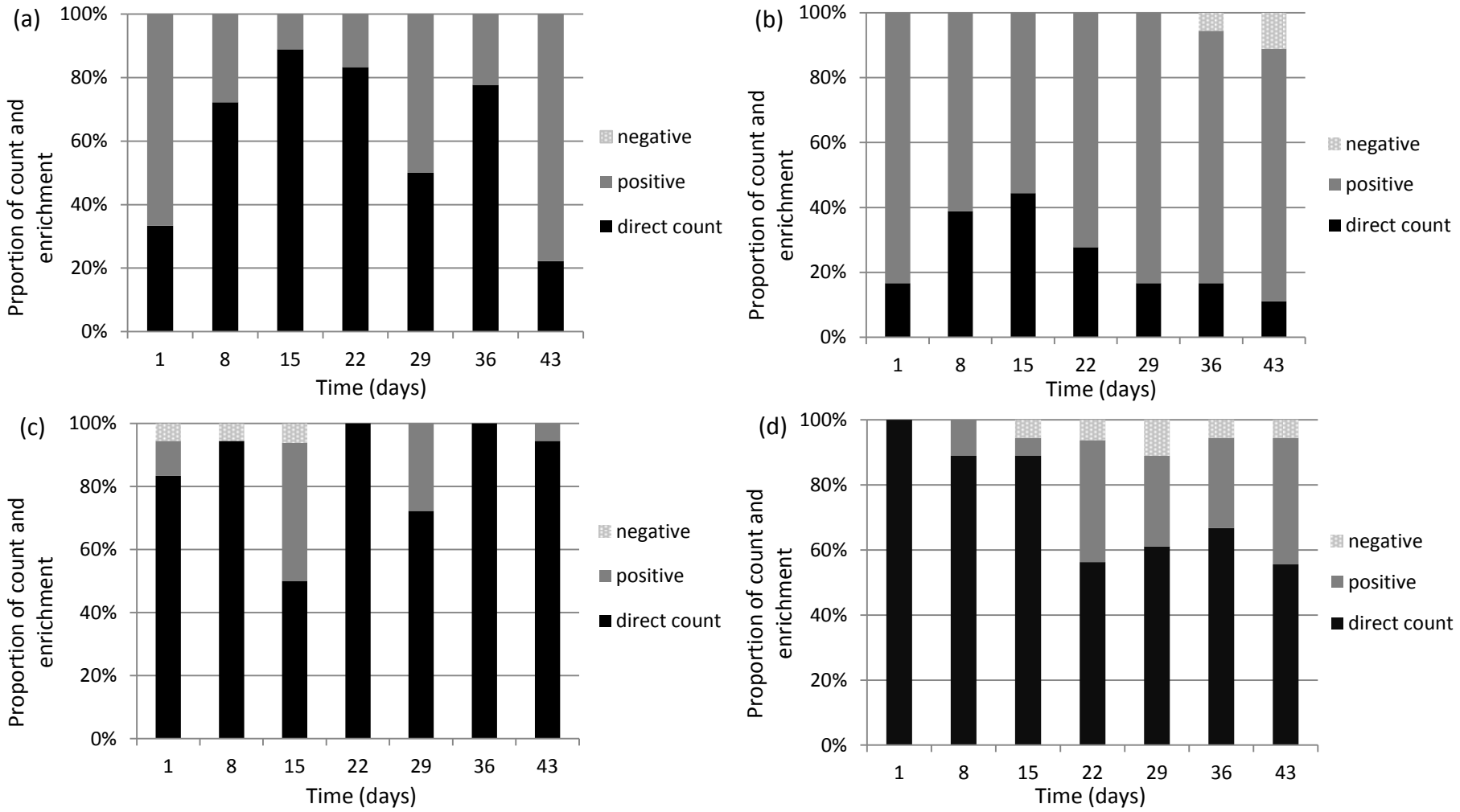


Figure 4.16 The proportion (%) of direct count and enriched *Salmonella* cocktail samples in (a) sandy soil with chicken manure, (b) sandy soil, (c) clay soil with chicken manure amendments and (d) clay soil. Data were pooled from two factors (temperature and moisture, n = 18).

4.4 Discussion

Salmonella has been reported to survive in soil or manure-amended soil from a few days to up to a year, with various environmental factors affecting its survival. In the present study, the interaction effects of two factors at a time were evaluated to predict the survival of *Salmonella* serovars in either a clay or sandy soil, with and without chicken manure amendments, using different temperatures, moisture regimes and serovars.

4.4.1 Effect of temperature on *Salmonella* survival

Temperature has been shown to be an important factor for the metabolism and survival of pathogenic bacteria in environmental samples (Himathongkham *et al.* 1999; Bovill *et al.* 2001; Arrus *et al.* 2006; Semenov *et al.* 2007). In the present study, a significant difference in *Salmonella* survival was observed at different temperatures over the 43 day incubation period. Generally, the survival of *Salmonella* at lower temperatures (5 and 21°C) was found to be greater than survival at higher temperature (37°C). The rate of *Salmonella* decline was 28% and 41% at 5 and 37°C respectively throughout the incubation period. The average rate of decline of *Salmonella* over 43 days of incubation period was 0.84, 0.80 and 1.30 $\log_{10}$ CFU g^{-1} at 5, 21 and 37°C, respectively. In addition, the effect size between 5 and 21°C and 21 and 37°C was found to be small but medium effect size was observed between 5 and 37°C. However, by day 43 there was no significant difference between the two temperature treatments with respect to the temperature-time interaction. This result is similar to the previous study of Zibilske and Weaver (1978) who reported the rapid decline of *Salmonella* cells in clay soil at 39°C. Semenov *et al.* (2007) also reported that the decline of bacterial pathogens was greater when the temperature was increased from 7 to 33°C. In general, survival rates of *Salmonella* spp. decline as temperatures are increased from 20 to 70°C in natural substrates (Kudva *et al.* 1998; Himathongkham *et al.* 1999). In the present study, the number of bacterial cells was found to be similar after incubation at both higher and lower temperatures based on the statistical tests of significance. This may be due to greater rates of death and regrowth of bacterial cells at higher temperatures while there was a lower rate of decline and less regrowth at the cooler temperature. Some survival studies report the rate of decline of bacterial pathogens is greater at lower temperatures (Jiang *et al.* 2002; Bach *et al.* 2005; Mukherjee *et al.* 2006a).

The interaction of temperature and soil was found to be significant for the survival of *Salmonella*. In all temperature treatments, survival was better in clay soil than sandy soil. In clay soil, the survival of *Salmonella* was better at lower temperature (5°C) than at higher temperature (37°C). This is in agreement with Garcia *et al.* (2010), who reported that the greatest decline in bacterial levels in the soil was measured at higher temperature (25°C) and corresponds with other publications (Holley *et al.* 2006; Moynihan *et al.* 2013; Li *et al.* 2015).

In the present study, for all temperature treatments the survival of *Salmonella* was better in the presence of manure than without manure. However, in the presence of manure, survival of *Salmonella* was better at higher temperature (21°C) than at lower temperature (5°C). On the other hand, without manure, survival of *Salmonella* was better at lower temperature (5°C) that at higher temperature (37°C).

There was a difference in the survival of the individual *Salmonella* serovars according to temperature. The survival of the *Salmonella* cocktail, *S.* Enteritidis and *S.* Montevideo was better at lower temperatures compared with higher temperatures. However, there were no significant differences between the temperature treatments for *S.* Sofia. The difference among *Salmonella* serovars may be related to the presence or absence of specific genes related to tolerance of environmental stress and their level of expression. It has been reported that *Salmonella* uses cold shock proteins (CSPs) as a response for quick adaptation to temperature downshifts in the environment (Craig *et al*. 1998; Jeffreys *et al*. 1998; Horton *et al*. 2000; Andina and Hanning 2015). It has also been reported that *Salmonella* has the ability to increase its survival rate by expressing CSPs when treated at low temperatures (5°C to 10°C). For example, *S.* Enteritidis is able to survive in chicken tissues at 2°C (Andino and Hanning 2015). There may well be a difference among *Salmonella* serovars in adapting to lower temperatures which may result in a difference in their survival pattern. Furthermore, soil type may interact with temperature to modify survival. In sandy soils, high temperature would hasten moisture loss resulting in an unsuitable environment for the bacteria. In clay soils however, the size and nature of the particles and minerals may enhance survival (Brennan *et al*. 2014).

4.4.2 Effect of soil type on *Salmonella* survival

The survival of *Salmonella* was found to be different between the two soil types (sandy and clay) with survival significantly better in clay soil than in sandy soil throughout the

microcosm pot trial. In addition, effect size between the two soil types was found to be large (d = 1.47). This result is in agreement with previous findings that sandy soils are more hostile in terms of survival of enteric organisms compared with clay soils (Lang and Smith 2007). Bacterial nutrition and the physical and chemical properties of the soil have been suggested as reasons for variation in survival of *Salmonella* in different soil types (Chandler and Craven 1980). For example, soils with high organic matter content and a fine texture have been found to support three times greater microbial populations than coarse textured soils (Tate 1978; Mubiru *et al.* 2000). In the present study, the amount of total carbon in the clay soil was approximately five times greater than in the sandy soil prior to chicken manure amendment. The increased organic matter and high water holding capacity could be the reason the clay soil resulted in better survival of *Salmonella* than the sandy soil.

Another property which has been found to affect the survival of bacterial pathogens is soil pH. It has been reported that survival of *Salmonella* and *E. coli* O157:H7 was affected by pH in which pathogen populations decreased more rapidly in acidic soils than in neutral or slightly alkaline soils (Erickson *et al.* 2014). In addition, Ma *et al.* (2013) indicated that pH was determined to be a major factor affecting the survival of *E. coli* O157:H7 in a study conducted in different soil types having a pH range from 6.7–8.0. In the present study, pH ranged from 5.06 (clay soil) to 7.53 (sandy soil with manure amendment) but the relationship between pH and *Salmonella* survival was found to be inconsistent and changes in soil pH were not monitored throughout the incubation period.

Electrical conductivity is another soil property affecting the survival of bacterial pathogens. The EC of soil is an indication of ion concentration (dissolved salts) in the soil solution. Increasing salinity, which results in an increase in EC, may cause interference in ion transport and inhibition of enzyme activity, both of which could lead to reduced survival of enteric pathogens in soils (Erickson *et al.* 2014). Adding manure to both soil types (clay and sandy) increased the EC but it cannot be used to account for the difference in survival of *Salmonella*.

In the present study, manure amendments improved the survival of bacteria in soil compared to non-amended soil samples most likely because of an increase in nutrient availability. Franz and van Bruggen (2008) reported that *E. coli* O157:H7 survival was lower when there were lower levels of readily available carbon in both manure and soil. Additionally, Barbour (2002) reported that counts of all microorganism increased significantly in soil treated with chicken manure. Santamaria and Toranzos (2003) reported that soluble organic compounds

increased survival and, in the case of bacteria, were thought to favour their regrowth. Several studies have also described the protective effects of moisture and soil organic matter on the survival of bacterial pathogens (Dowe *et al.* 1997; Cools *et al.* 2001; Holley *et al.* 2006) and soil rich with organic matter has greater capacity to retain other nutrients (Jamieson *et al.* 2002). Franz *et al.* (2008a) compared the survival of *E. coli* O157:H7 in soil amended with farmyard manure (compost) and artificial fertiliser and reported that survival was higher in compost-amended soil whereas the pathogen survived for a shorter period of time in soil amended with artificial fertiliser. Nutrient availability provided by addition of manure may allow cell repair leading to greater numbers of *Salmonella* (Holley *et al.* (2006).

4.4.3 Effect of soil moisture on *Salmonella* survival

Another abiotic factor affecting soil microbial communities is moisture. The importance of this factor is highlighted as soil moisture content has a greater impact on bacterial comunities than soil nitrogen or carbon (Singh *et al.* 2009). Generally, a decrease in soil moisture has a direct effect on pathogen decay due to desiccation and autolysis of bacterial cells (Lang and Smith 2007). In the present study, *Salmonella* survival was found to be better in constant moisture conditions than soil exposed to fluctuating moisture levels such that the average *Salmonella* count at the end of the constant moisture incubation period was 2.24 and 3.92 $\log_{10}$ CFU g^{-1} in sandy and clay soils, respectively. In contrast, the mean *Salmonella* count at the end of the incubation period fluctuating moisture was half these values (i.e. 1.46 and 2.94 $\log_{10}$ CFU g^{-1} in sandy and clay soil, respectively). In addition to ANOVA, effect size was calculated and large effect size (d = 0.81) was observed between constant and fluctuating moisture regimes (data not shown). Survival of bacterial pathogens has been reported to increase when the soil is moist (Entry *et al.* 2000) and it has also been shown that bacterial survival is greater in finer grained soils, which have an enhanced ability to retain moisture and nutrients (Jamieson *et al.* 2002). The interaction of moisture and temperature was significant with better survival at lower temperatures in soil with constant moisture. This result is in agreement with Entry *et al.* (2000) who found that the survival rate of bacterial pathogens decreases when higher temperatures are combined with drying.

The better survival of *Salmonella* in clay soils is also reflected in the *Salmonella* presence/absence data where the proportion of samples counted by direct plating was 94% and 55%, in manure-amended and unamended clay soil, respectively, and only 22% and 11% in manure-amended and unamended sandy soil, respectively. Only 6% of the samples were

enriched to check the viable residual *Salmonella* cells in clay soil with manure-amended treatments and this pattern was supported by the plate count data in which the survival of *Salmonella* was better in manure-amended clay soils. The low counts for *Salmonella* in clay soil however may not pose a safety risk to human health as 10^{-5}–$l0^{-10}$ organisms are required as the infection dose depending on serovar and health of the individual (Kothary and Babu 2001).

Based on the present study, vegetables growers can expect faster die off of *Salmonella* in contaminated fields, particularly in sandy soil in summer when the soil temperature is higher and is coupled with a fluctuating moisture regime. These conditions provide an advantage for the growers where the soil temperature, air temperature and evaporation are high. In clay soil, a longer period of time between the application of improperly composted or fresh manure and planting of vegetables is required to reduce risk of *Salmonella* contamination of crops, particularly in winter when soil temperatures are low. The addition of manure enhanced the survival of *Salmonella*, which is an issue since most growers want to use organic amendments (manure or compost) to increase the soil carbon. It is unlikely, however, that organic amendments will be replaced with inorganic inputs under sustainable crop production systems (Quilty and Cattle 2011). As a result, the different treatments used to suppress *Salmonella* contaminated soil by simulating accidental chicken manure application under field condition in relation to soil types, soil and weather conditions will be discussed in Chapter 5.

4.4.4 Summary

Overall, the survival of *Salmonella* was found to be better in clay soil than sandy soil. Sand-based soils are more hostile in terms of survival of enteric organisms. Soil types with different physical and chemical properties can also differ in terms of bacterial nutrition which contributes to the survival of enteric organisms. The addition of manure to soil increases the availability of soil organic carbon, nitrogen and other nutrients which can enhance the survival of bacterial pathogens. In the present study, manure amendments improved the survival of *Salmonella* in soil compared with non-amended soils. In addition, environmental factors such as temperature and moisture were found to be important in affecting the survival of bacterial pathogens in soil and manure-amended soil. Generally, the survival of *Salmonella* was found to be greater at lower temperature (5°C) that at higher temperature (37°C).

Regarding moisture, *Salmonella* survival was found to be better in constant moisture conditions compared to the fluctuating moisture regime in both sandy and clay soils.

Based on the present study, addition of raw manure to clay soil is not recommended with a short-cycle vegetable crop due to the slow die off rate of *Salmonella*, particularly in winter. Use of certified composted manure is recommended.

5. Field experiment: remediation of *Salmonella*-contaminated soil using cover crops and solarisation

5.1 Introduction

It is only in the last thirty years that there has been a rise in frequency of *Salmonella* outbreaks linked with the consumption of contaminated fresh produce (Abd-Elall and Maysa 2015). In the USA, fresh produce is estimated to cause 20 million illnesses costing $38.6 billion every year (Olaimat and Holley 2012). In particular, lettuce is a commonly reported cause of fresh produce disease outbreaks due to enteric pathogen contamination because it is consumed raw and is a major component of ready-to-eat salad products (James 2007). In Australia in 2001, 41 cases of *Salmonella* infection were associated with contaminated lettuce (Stafford *et al.* 2002).

Contamination of salad vegetables may occur either preharvest (Islam *et al.* 2004c) or postharvest (Sánchez *et al.* 2012), although it has been reported that the most scrutiny of microbiological quality and safety of finished products should occur during the preharvest stage (Beuchat 1996; Brackett 1999; Beuchat 2002; James 2007). Preharvest causes of contamination are the use of raw or partially composted manure, contaminated irrigation water or intrusion of animals in the field (Bernstein *et al.* 2007b; Berger *et al.* 2010). For example, in Australia, poultry litter and raw manure, a by-product of the broiler and egg production industries, are widely applied to commercial vegetable production sites as soil amendments. Raw or partially composted animal manures are known to harbour enteric pathogens such as *Salmonella*, *E. coli* and *Listeria monocytogenes.* So application of these manures may introduce pathogens into soils used for vegetable production (Natvig *et al.* 2002; Franz *et al.* 2005).

Once in the field, bacterial pathogens may survive and multiply if conditions are favourable increasing the risk of produce contamination (Park *et al.* 2012). Abiotic factors such as temperature, pH, soil moisture and soil type, together with biotic factors such as microbial community affects whether bacteria, originating from manures or composts, will persist (van Veen *et al.* 1997). Soil type has been identified as an important variable in pathogen survival; clay soil supports greater survival of enteric pathogens than sandy soils (Danyluk *et al.* 2008). This was also found in the present study (Chapter 4), in which clay soil supported the

survival of *Salmonella* more than sandy soil. In addition, other factors such as low temperatures and constant soil moisture favour the long-term survival of *Salmonella*, thereby increasing the risk of vegetable contamination, either through direct contact with the contaminated soil, or through irrigation and rain splash.

Bacterial pathogens in soil or manure-amended soil can be suppressed by biocidal compounds that possess antimicrobial activity as described in Chapter 2. Biofumigation is an agronomic strategy to control pathogens, whereby green manure crops are incorporated into the soil and release biocidal compounds as they degrade (Kirkegaard *et al.* 1997; Matejiceck *et al.* 2002; Gimsing *et al.* 2005). Glucosinolates are a group of compounds produced by plants in the family Brassicaceae that are responsible for the characteristic pungent flavours in crops such as mustard, radish and cabbage (Gimsing *et al.* 2005). The Brassicaceae plant species that are generally considered for biofumigation are *Brassica oleracea* (broccoli, cabbage, cauliflower, kale), *Raphanus sativus* (radish), and various mustards, such as *Sinapis alba* (white mustard), *Brassica carinata* (Ethiopian mustard) and *Brassica juncea* (Indian mustard) (Sarwar *et al.* 1998; Ploeg 2008).

Isothiocyanates (ITCs) are sulfur-containing hydrolysis products of glucosinolates (Wathelet *et al.* 2004) and are generally considered the most toxic group of secondary metabolites in this family (Gimsing *et al.* 2005). In addition, oxazolidinethiones, nitriles and thiocyanates are some of the other products of glucosinolate hydrolysis (Kirkegaard *et al.* 1997). Sulforaphane from *Brassica* has shown antibacterial activity against a range of both Gram-negative and Gram-positive bacteria (Aires *et al.* (2009). Many studies have indicated that ITCs exhibit antimicrobial activity against diverse organisms including fungi, bacteria, and insect pests. In particular, allyl ITC from sinigrin (2-propenyl glucosinolate), exhibits antimicrobial activity against a variety of pathogens at low concentrations (Luciano and Holley 2009; Liu and Yang 2010). Similarly, Lin *et al.* (2000) reported the antibacterial activity of methyl ITC against *S.* Montevideo, *E. coli* O157:H7 and *L. monocytogenes* in fresh produce.

Another group of antimicrobial plant compounds possessing a biofumigant effect against bacterial pathogens are the phenolics (Cetin-Karaca 2011). For example, the phenolic compound, ellagitannin, from red raspberry has antimicrobial properties against the growth of human pathogens (Heinonen 2007).

In the present study, 'Terranova' oilseed radish (*Raphanus sativus*) and 'Cappuccino' Ethiopian mustard (*Brassica carinata*) were used as a source of glucosinolates. 'Fumig8tor' sorghum was used as a source of phenolics for the biofumigation treatments for suppressing *Salmonella* in the soil. According to Gutierrez and Perez (2004), sap from radish root showed antimicrobial activity against *Bacillus subtilis*, *Pseudomonas aeruginosa* and *Salmonella thyphosa.* The compound 3-thioxo-3-pyrrolidinecarbaldehyde (TPC) is a major component of radish, and possesses antimicrobial activity against fungi and bacteria with a minimum inhibitory concentration ranging from 50–400 µg ml^{-1}. In one study, *S.* Typhimurium showed a higher sensitivity against TPC at a concentration of 200 µg ml^{-1} (Matsuoka *et al.* 1997). Turgis *et al.* (2009) reported that an essential oil extracted from mustard has been found to reduce intracellular ATP concentration both in *E. coli* O157:H7 and *Salmonella*.

Soil solarisation is a relatively common pre-planting technique used to control pathogens and pests. The soil is covered with polyethylene plastic sheeting for a certain period of time and the resultant heat can inactivate bacterial and fungal pathogens in the soil (Stapleton 2000; Barbour 2002; Berry and Wells 2012). It is practiced mostly in summer when high ambient temperatures can exceed 40°C. In Australia, black plastic is widely used for soil solarisation and the same was used in the present study as a means to increase soil temperature. Soil solarisation in combination with biofumigation may synergistically control microorganisms in the soil. For example, according to Gamliel and Stapleton (1993), the amount of volatile compounds in soil amended with cabbage residue was higher in heated (solarised) soil than in the corresponding unheated soil. However, there has been little research conducted on these processes as potential techniques for supressing *Salmonella* in contaminated fields under Australian conditions. Additionally, even though *L. monocytogenes* is not the primary focus of this study, it is critical to determine the potential for the target remediation treatments to elevate the population of the other bacterial pathogens in the soil. This pathogen is recognised as a soil resident and prevalent coloniser of decaying vegetation in agricultural systems and transfer to fresh produce has become an increasing concern (Hoelzer *et al.* 2012). Therefore, the present study was initiated with the following aims:

1. Determine the optimal low-residue cover crop that will enhance die-off of *S. enterica* in contrasting soils in Australia.
2. Establish single or combined cover crop-solarisation combinations that will facilitate die-off of *S. enterica* in soil so that there is no re-contamination associated with the re-planting of leafy greens.

3. Detect *L. monocytogenes* in cover crop-amended soils under field conditions.

5.2 Materials and methods

5.2.1 Site description

The experiment was conducted at two research sites, Karalee Farm and Pye Farm at the University of Sydney. These research sites are described in detail in Chapter 3.1.

5.2.2 Soil description

The chemical and physical properties of the soil were analysed as described in Chapter 3.2.3.

5.2.3 Baseline characterisation of field soil

To check for the presence or absence of *Salmonella* and *L. monocytogenes*, random soil samples were taken from field plots established at Karalee Farm and Pye Farms (n = 10 per site, an approximate land area was 15 m wide and 65 m long per site; see Fig. 5.1). For detection of *Salmonella*, 100 g of each soil type was transferred to 400 mL sterile stomacher bags and aliquots of 150 mL BPW was added to each bag and incubated for 24 h at 37°C. An aliquot (10 mL) of this sample was then transferred to 90 mL of TTB containing an iodine supplement and incubated for 6 h at 42°C. Samples were then treated as described in Chapter 3.6.3.

To check for the presence or absence of *L. monocytogenes*, the protocol described in Chapter 3.6.4 was followed.

5.2.4 Baseline characterisation of commercial chicken pellet samples

Chicken manure pellets were obtained from a commercial supplier (as described in Chapter 3.6.4) and used as an inoculum carrier and as a soil amendment. The chicken manure pellets from commercial suppliers are typically composted, heat treated and extruded into pellets. Baseline characterisation of the chicken manure pellets for *Salmonella* was done prior to field application. Replicate samples (100 g) of chicken manure pellets (n = 3) were weighed and transferred to 400 mL sterile stomacher bags. Aliquots of 150 mL of BPW was added to each bag and incubated for 24 h at 37°C. An aliquot (10 mL) of incubated BPW sample was transferred to 90 mL of TTB containing an iodine supplement and incubated for 6 h at 42°C. Samples were then treated as described in Chapter 3.6.3.

The chicken manure pellet samples were also checked for the presence or absence of *L. monocytogenes* following the protocol described in Chapter 3.6.4.

5.2.5 Preparation of field sites

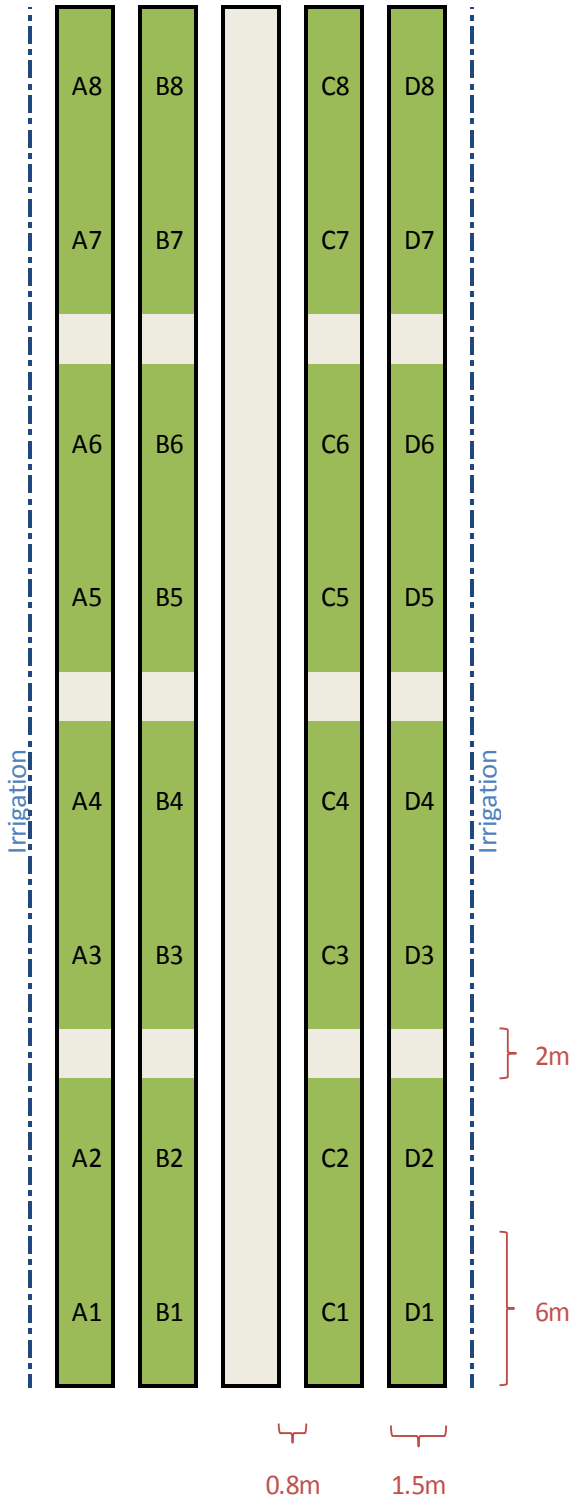
The experimental design was a split-plot block design with the whole plot treatment being type of cover crop (mustard, radish, sorghum and no cover crop) and the split plot treatment being solarisation or no solarisation.

At both research field sites, five raised beds were formed with a 1.5 m bed width, where the central bed was left undisturbed as a buffer zone (see Figure 5.1). The length of each whole bed was 12 m with 2 m row breaks and 0.8 m wheel tracks. Plot areas were marked out with stakes and marker spray.

5.2.6 Preparation of inoculum

Salmonella inoculum was prepared from lawn cultures grown on TSARP at 37°C for 24 h. The cultures were washed with 5 mL phosphate buffer, rubbed with an L-shaped spreader and the resultant suspension aseptically transferred to a sterile bottle. Initially the inoculum was made from 20 lawn cultures, but this was found to provide an insufficient level of contamination when applied to chicken manure pellets. Hence, the pellets were inoculated a second time using inoculum prepared from 100 lawn cultures. The bottle was stored at 4°C until inoculation of the chicken manure pellets on the same day. Approximately 7 $\log_{10}$ CFU *Salmonella* per gram of chicken manure was used to inoculate the pellets which is in the range of the reported density of enteropathogens in a natural condition which is from 10^4 CFU g^{-1} dry-matter to 10^7 CFU g^{-1} dry-matter of manure (Semenov *et al.* 2007).

Rifampicin-resistant *Salmonella* was used because markers such as antibiotic resistance aid in the selection and enumeration of target pathogens from manure-amended soils which generally contain high populations of background microorganisms (Harris *et al.* 2013).



Plot coordinates		Treatment	
Block	**Row**	**Whole-plot**	**Split-plot**
A	1	Mustard	Solar
A	2	Mustard	Open
A	3	No Cover	Solar
A	4	No Cover	Open
A	5	Sorghum	Solar
A	6	Sorghum	Open
A	7	Radish	Open
A	8	Radish	Solar
B	1	Mustard	Open
B	2	Mustard	Solar
B	3	No Cover	Open
B	4	No Cover	Solar
B	5	Radish	Solar
B	6	Radish	Open
B	7	Sorghum	Solar
B	8	Sorghum	Open
C	1	No Cover	Solar
C	2	No Cover	Open
C	3	Radish	Open
C	4	Radish	Solar
C	5	Mustard	Open
C	6	Mustard	Solar
C	7	Sorghum	Solar
C	8	Sorghum	Open
D	1	No Cover	Open
D	2	No Cover	Solar
D	3	Mustard	Open
D	4	Mustard	Solar
D	5	Radish	Open
D	6	Radish	Solar
D	7	Sorghum	Solar
D	8	Sorghum	Open

Figure 5.1 Field layout and treatment combinations at Karalee Farm, Camden and Pye Farm, Bringelly. Field design diagram provided by Dr Kim-Yen Phan-Thien, University of Sydney.

The serovar intended for use in this experiment was rifampicin-resistant *S.* Sofia because it is avirulent and the most prominent serovar in Australia. The identity of the serovar was confirmed by a positive agglutination slide test using O:4 (B-group) antiserum. However, at a late stage of the field experiment, soil samples were found to react positively with O:7 (C1 group) antiserum. Genetic sequencing by an analytical service laboratory confirmed that the samples contained *S.* Montevideo and that the original inoculum had contained a mixture of *S.* Sofia and *S.* Montevideo. This was not ideal and reflects technical inexperience. However, the experiment itself followed a robust design, the results remain valid since the difference between *S.* Sofia and *S.* Montevideo was not statistically significant on time-serovar and temperature-serovar interactions except at 5°C and provide insights into *Salmonella* population dynamics in response to the field treatments.

Before inoculation, the preparation area was cleaned and thoroughly disinfected using 75% ethanol and 1% decontaminant (Virkon) solution. The inside of all containers used to mix or hold chicken manure pellet with liquid inoculum was disinfected using 75% ethanol and not a sanitiser to avoid residues. The plastic boxes used to hold the chicken manure pellets, the cement mixer used to mix the liquid inoculum and the chicken manure pellets and the watering can used to transfer the liquid inoculum to the cement mixer were all washed using tap water, disinfected using 75% ethanol (approximately 2 L) and air-dried before use.

A total of 160 kg of chicken manure pellets obtained from a commercial market (Enfield Produce, Pet and Garden Supplies, Sydney, Australia) were prepared for both farm research sites. The pellets were divided into approximately 20 kg lots for ease of management. A volume of 625 mL of bacterial suspension was mixed with 2 L dechlorinated tap water. The diluted suspension was showered onto 20 kg of chicken manure pellets while rotating in a conventional cement mixer. The inoculated pellets were divided into two plastic crates and loosely covered with a plastic lid to enable drying.

There were a total of eight boxes containing inoculated chicken manure pellet and a composite sample was taken from each 20 kg pellet mix (per box) to determine the concentration of *Salmonella* cells. The extraction and enumeration (n = 8) were done following the protocol described in Chapter 3.6.2. After the initial inoculation, the concentration of *Salmonella* in the chicken manure was approximately 5.0 $\log_{10}$ CFU g^{-1} of manure. This was lower than desired, so the manure was inoculated a second time with a

more concentrated inoculum to achieve a contamination level of about 7.0 $\log_{10}$ CFU g^{-1} (Table 5.1).

Table 5.1 *Salmonella* concentration in inoculated chicken manure pellets enumerated on rifampicin-amended trypticase soy agar (TSARP) and xylose lysine deoxycholate (XLDRP) plates. Values are mean ± standard error, n = 8. CFU = colony forming unit.

Media	***Salmonella* count ($\log_{10}$ CFU g^{-1})**	
	First inoculation	**Second inoculation**
TSARP	5.82 ± 0.10	7.18 ± 0.06
XLDRP	5.67 ± 0.01	7.07 ± 0.05

During the field experiment the following biosafety practices were performed:

- Hands (before and after sampling) were wiped with 75% ethanol and working areas were disinfected with 1% virkon
- Placed warning signs of biohazard on equipments, sampling and sample storing boxes etc. *Salmonella* samples were stored in double container and labeled properly
- Wore overalls and use gloves and masks while working with *Salmonella* cultures, inoculated chicken manure pellets and during sampling in the field.
- After sampling, disinfected the outsole and upper of shoes using 1% virkon or wore plastic shoe cover during sampling
- After sampling, disinfected all sampling equipment using 75% ethanol or 1% virkon

5.2.7 Application of chicken manure pellets to the field

Inoculated chicken manure pellets were applied to the designated treatment plots (2 days after inoculating the chicken manure pellet) at a commercial standard rate of 200 g m^{-2} in the top 5 cm of soil and incorporated manually using hand rakes (Figure 5.2). Samples were collected from whole plots immediately following application of the inoculated chicken manure at both research sites.

Figure 5.2 Application of rif-resistant *Salmonella*-inoculated chicken manure pellets at Karalee Farm, Camden.

5.2.8 Planting of lettuce

Green Oakleaf lettuce seedlings obtained from a commercial supplier were transplanted into the field plots one to two days after application of the inoculated manure. The seedlings were planted in two rows in each bed (excluding the buffer zone) at a spacing of approximately 30 cm. A mechanical transplanter was used at Karalee Farm, but seedlings at Pye Farm had to be manually transplanted due to the high clay content of the soil. Almost 90% of the seedlings at Pye Farm died and were replanted four days after the initial planting. Lettuce seedlings were allowed to grow for 4 weeks prior to being ploughed in and incorporated into the soil using a rotary hoe.

5.2.9 Application of cover crop treatment

Following lettuce incorporation, cover crop treatments were applied. The cover crops used in the present study were 'Terranova' oilseed radish (*Raphanus sativus*) and 'Cappuccino' Ethiopian mustard (*Brassica carinata*) donated by Seedforce, Australia and 'Fumig8tor' sorghum donated by Pacific Seeds, Australia. The cover crops were sown by hand at the commercially recommended rates (i.e. 1.0, 1.5 and 3.1 g m^{-2} for radish, mustard and sorghum, respectively). Plots were irrigated immediately after sowing and thereafter as

required. Cover crop treatments were incorporated into the soil 35 days after sowing by disking.

5.2.10 Application of the solarisation treatment

Black plastic is commonly used in Australian horticultural industries for weed control as well as solarisation (Rogers *et al*. 2002). For this reason, black plastic (Austec Irrigation and Garden Supplies Pty Ltd, Narellan, NSW) was used in this experiment, although some research suggests that clear plastic is more effective in raising soil temperature (Stapleton and DeVay 1986).

Solarisation treatments were applied by covering the soil surface (after irrigation) with black polyethylene sheets (6 x 4 m; 200 µm thickness) and digging in the edges to achieve a tight fit (Figure 5.3). In the control whole plots (no cover crop), solarisation was applied at the time that cover crops were sown in other whole plots. In the whole plots treated with cover crops, solarisation was applied after the cover crops were ploughed in. All whole plots comprised a split plot with solarisation treatment and a control split plot without solarisation.

Figure 5.3 The soil solarisation treatment and cover crop at Karalee Farm prior to crop incorporation into the soil at Karalee Farm, Camden.

5.2.11 Weather and soil conditions

The temperature of the soil (surface and 5 cm depth) was automatically recorded at hourly intervals using Thermochron iButton temperature loggers (Thermodata Pty Ltd, Australia) throughout the experimental period at both field sites. Temperature was recorded for both solarised and non-solarised plots. Soil moisture of both sites was measured every week at the time of soil sampling using a soil moisture meter (MP406 Moisture Probe, Instrument Choice, Australia). Weather data was obtained from Bureau of Meteorology weather stations closest to the field sites (i.e. Camden and Badgerys Creek).

5.2.12 Soil sampling and transportation

Soil sampling was performed on a weekly basis during the experiment. Soil was sampled by pushing a sterile stainless steel cylindrical core (5 cm diameter, 10 cm depth) into the soil and carefully extracting. Before application of treatments (ploughing in of the cover crops and covering the soil with black plastic), random sampling was performed on each whole plot and, after the treatments started, sampling was performed from each split plot. Sampling from solarised plots was performed by cutting the plastic in an L-shape (Figure 5.4), removing the soil and sealing the cut using cloth tape. Each soil sample was a composite of three subsamples from each plot taken from the middle of the plots. The soil samples were placed in sterile 400 mL bags, and thoroughly massaged and mixed by hand to obtain a homogenous sample. The remaining soil left in the sampling bag was sealed and kept on ice and transported to the laboratory for the extraction of *Salmonella* and *L. monocytogenes* the next day after sampling.

5.2.13 Sample extraction and enumeration

To extract *Salmonella*, 100 g of soil from the composite sample was aseptically transferred to a 400 mL sterile stomacher bag using a sterile spoon, and 100 mL of extraction buffer was dispensed into the sample bag using a gravimetric dilutor. All extractions and mixing were performed following the steps described in Chapter 3.6.2.

5.2.14 Enrichment steps for *Salmonella*

Soil samples in which *Salmonella* was undetectable by direct plate count ($\leq$15 CFU per g soil) were retested following enrichment to determine whether the *Salmonella* cells survived at a very low level in the soil. The minimum level of detection on plates was calculated as the

reciprocal of the dilution factor (100 g soil and 150 mL extraction buffer) multiplied by a correction factor to report per mL (1 CFU per 100 µL or 10 CFU per mL). A 145 mL aliquot of the original suspension remaining after taking the 5 mL aliquot for enumeration was enriched with 145 mL of double strength BPW and incubated at 37°C for 24 h. Enrichment was done following the steps described in Chapter 3.6.3.

Figure 5.4 Tape-sealed L-shape cut after sampling soil from the solarisation plots at Karalee Farm, Camden.

5.2.15 Enrichment steps for *Listeria monocytogenes*

The presence/absence of *L. monocytogenes* in the soil sample was detected by following the method described in Chapter 3.6.4.

5.2.16 Statistical analysis

Microbial count data was log-transformed and analysed using a mixed model in JMP Pro version 11 (SAS 2014, Cary, NC, USA). The split-plot, whole-plot and the time of sampling were considered as fixed effects, while block was considered as a random effect in the model. Because whole-plot and split-plot treatments were applied at different stages of the experimental period, the data were analysed as three subsets: (1) before application of any treatment (day 0–32); (2) during the cover crop growth period (day 33–67) and application of

solarisation for controls; and (3) after the incorporation of the cover crops and application of solarisation treatment (day 68–105).

The first subset was analysed to examine change in *Salmonella* population over time. The second subset was analysed to examine the effects of time, fallow-solarisation and cover crop treatments (during their growth) on the *Salmonella* population. The third subset was analysed to examine the effects of time, cover crop treatments (after incorporation), cover crop-solarisation and fallow-solarisation treatments on the *Salmonella* population.

In addition, to compare the two soil types, a subset of data (the first 5 weeks) from Karalee Farm (sandy soil) was log-transformed and analysed in the same way as described above. When there were significant differences between the factors, a post-hoc test was done using Tukeys significant difference test for multiple comparisons. An example of this method of analysis is given in Appendix 3. The presence/absence data for *Salmonella* and *L. monocytogenes*, which were obtained by sample enrichment, were not statistically analysed as nearly all the results were found to be positive.

5.3 Results

5.3.1 Detection of *Salmonella* and *Listeria* in chicken manure pellets and field soil

The chicken manure pellets and field soil were evaluated to provide a baseline characterisation prior to the start of experiments. Neither the chicken manure or soil samples contained *Salmonella* but both sample types were found to be positive for *L. monocytogenes* after enrichment.

5.3.2 Soil characteristics

Soil samples from Pye Farm (clay loam referred to as ‘clay soil’) and Karalee Farm (coarse sand referred to as ‘sandy soil’) were analysed to determine the physical and chemical properties of soil at both field sites (Table 5.2).

Each soil had a different texture with varying amounts of sand and clay. The clay soil had four times more organic carbon than the sandy soil. Similarly, the amount of nitrate in the clay soil was seven times greater than the amount present in the sandy soil. In addition, the amount of total nitrogen and sulfur in the clay soil was seven and four times greater than the

amount found in sandy soil, respectively. Generally, the clay soil was richer than the sandy soil in terms of most nutrients (Table 5.2).

5.3.3 Weather and soil conditions

Soil moisture fluctuated according to rainfall events (data obtained from Bureau of Meteorology) and application of irrigation (Figure 5.5). Soil moisture was significantly (P <0.001) higher in clay soil than in the sandy soil for the duration of the experiment except on Day 63.

The relationship between soil moisture, measured on weekly basis during the time of sampling and weekly average precipitation, at the research site during the field experiment showed a strong correlation (r = 0.6, data not shown) between the soil moisture and precipitation. The field experiment started in mid-January which is mid-summer in Australia. The maximum daily air temperature reached approximately 36°C at Pye Farm and 37°C at Karalee Farm in the month of January (Figures 5.6 and 5.7). The mean maximum temperatures were 29°C and 29°C at Karalee and Pye farms, respectively (Table 5.3). The extent of day and night air temperature fluctuation ranged from 4–18°C and 3–17°C at Karalee and Pye farms, respectively.

Table 5.2 Physicochemical properties of soil obtained from the two research field sites. Values are mean ± standard deviation, n = 10. Values for which no error is presented were obtained from bulked soil samples analysed by a commercial laboratory using standard methods.

Variable	**Pye farm**	**Karalee farm**
Bulk density (g cm^{-3})	1.12 ± 0.03	1.28 ± 0.09
Soil moisture (%)	0.19 ± 0.05	0.05 ± 0.02
Colour	Brown	Light grey
Gravel (%)	0	0
Coarse sand (%)	10	64.5
Sand:silt:clay	27:27.1:35.9	27.7:2.6:5.2
Texture	2.0	2.0
Ammonium (mg kg)	9	1
Nitrate (mg kg)	98	14
Total nitrogen (%)	0.369 ± 0.063	0.048 ± 0.009
Phosphorus Colwell (mg kg)	112	33
Potassium Colwell (mg kg)	650	91
Sulfur (mg kg)	9.4	2.1
Organic carbon (%)	3.80	0.80
Conductivity (dS m)	0.238	0.037
pH ($CaCl_2$)	4.8	5.0
pH (H_2O)	5.4	5.8
Copper (mg kg)	4.17	1.00
Iron (mg kg)	385.70	84.01
Manganese (mg kg)	42.13	3.74
Zinc (mg kg)	12.69	1.97
Aluminum (meq 100 g)	0.282	0.093
Calcium (meq 100 g)	8.46	1.40
Magnesium (meq 100 g)	6.32	0.58
Potassium (meq 100 g)	1.24	0.22
Sodium (meq 100 g)	0.33	0.02
Boron (Hot $CaCl_2$ mg kg)	0.81	0.24

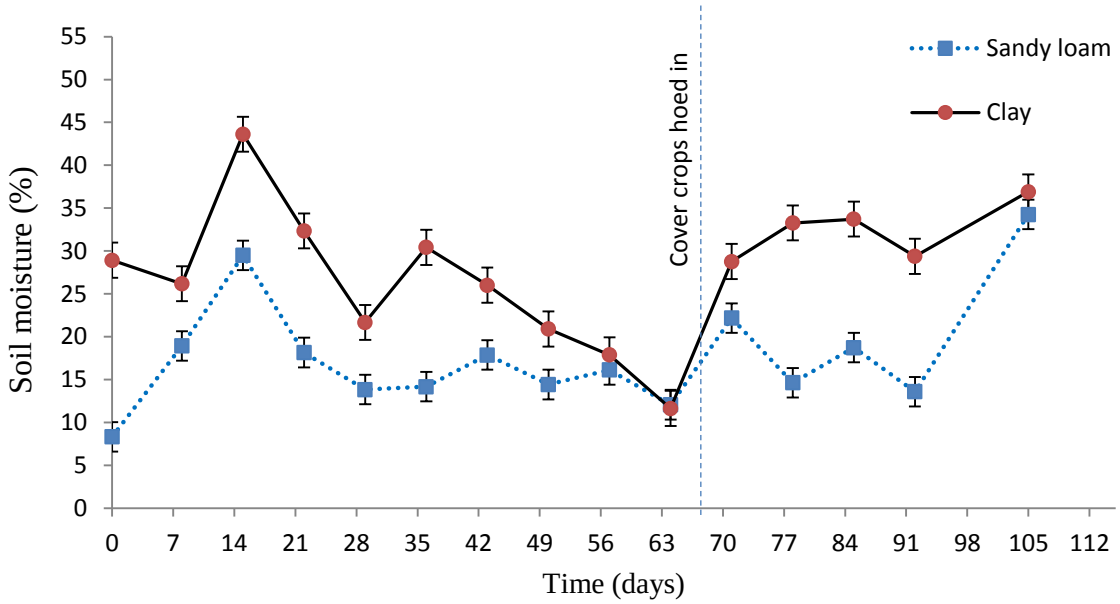


Figure 5.5 Soil moisture (%) at Karalee Farm, Camden (sandy soil) and Pye Farm, Bringelly (clay soil) over time. Data points are mean ± standard error, n = 20.

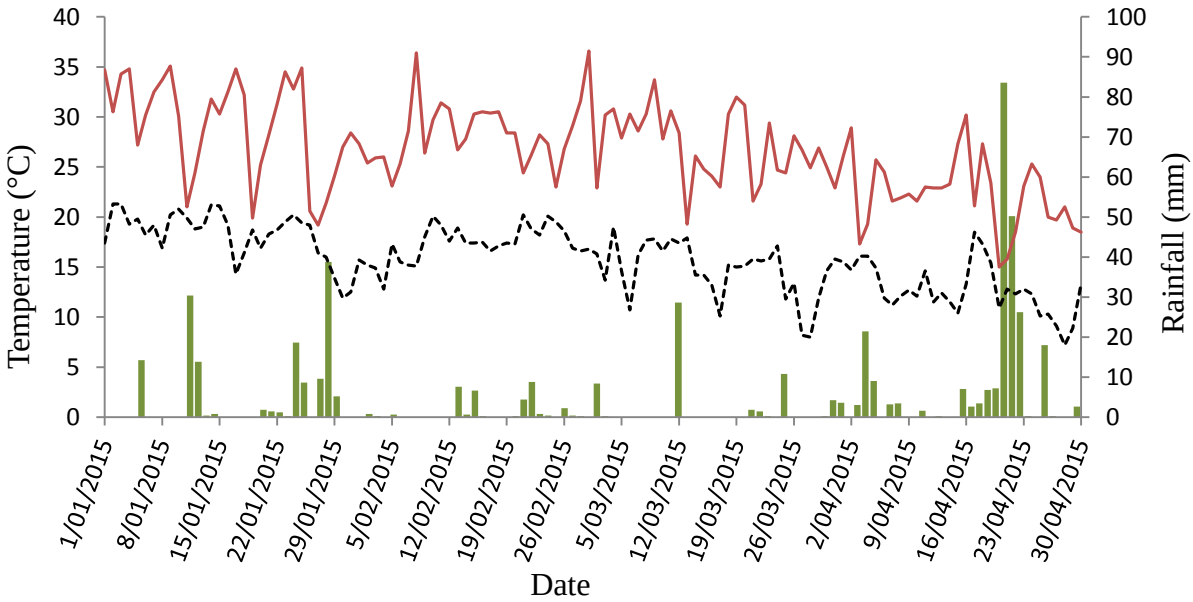


Figure 5.6 Daily weather conditions at Pye Farm, Bringelly during the field experiment. Data from Badgerys Creek weather station, Bureau of Meteorology. The black dotted line is minimum daily temperature (°C), the red solid line is maximum daily temperature (°C) and the green bars are daily precipitation (mm).

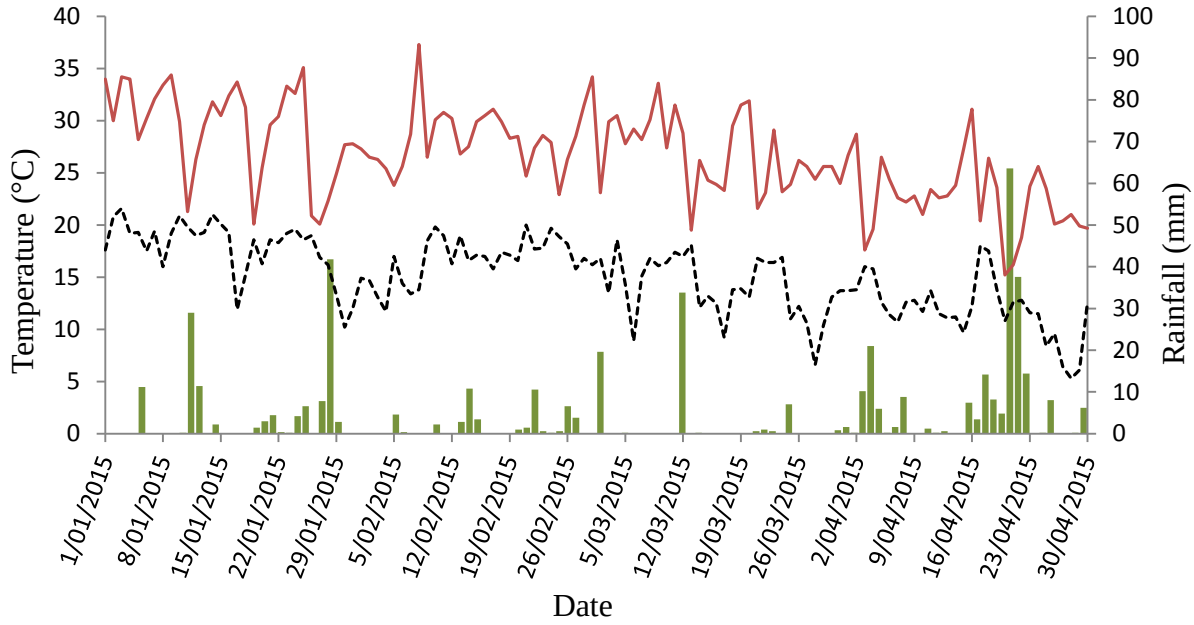


Figure 5.7 Daily weather conditions at Karalee Farm, Camden during the field experiment. Data from Camden weather station, Bureau of Meteorology. The black dotted line is minimum daily temperature (°C), the red solid line is maximum daily temperature (°C), and the green bars are daily precipitation (mm).

Table 5.3 Mean maximum and daily minimum temperatures (°C) and rainfall (mm) at the two sites (Karalee farm and Pye farm) during the field experiment.

	Minimum temperature (°C)		**Maximum temperature (°C)**		**Total rainfall (mm)**	
Month	**Karalee Farm**	**Pye Farm**	**Karalee Farm**	**Pye Farm**	**Karalee Farm**	**Pye Farm**
January	17.9	18.2	29.3	29.2	126.6	145.0
February	16.7	17.3	28.2	28.1	49.0	34.0
March	14.1	14.8	27.0	27.3	63.8	55.8
April	11.9	12.7	22.6	22.3	219.8	253.4

The solarisation treatment significantly increased the soil temperature, both at the surface and at 5 cm depths compared with the control treatment (Figure 5.8 and 5.9). There was also a significant difference in soil temperatures between the surface and 5 cm depths at both sites (Table 5.4).

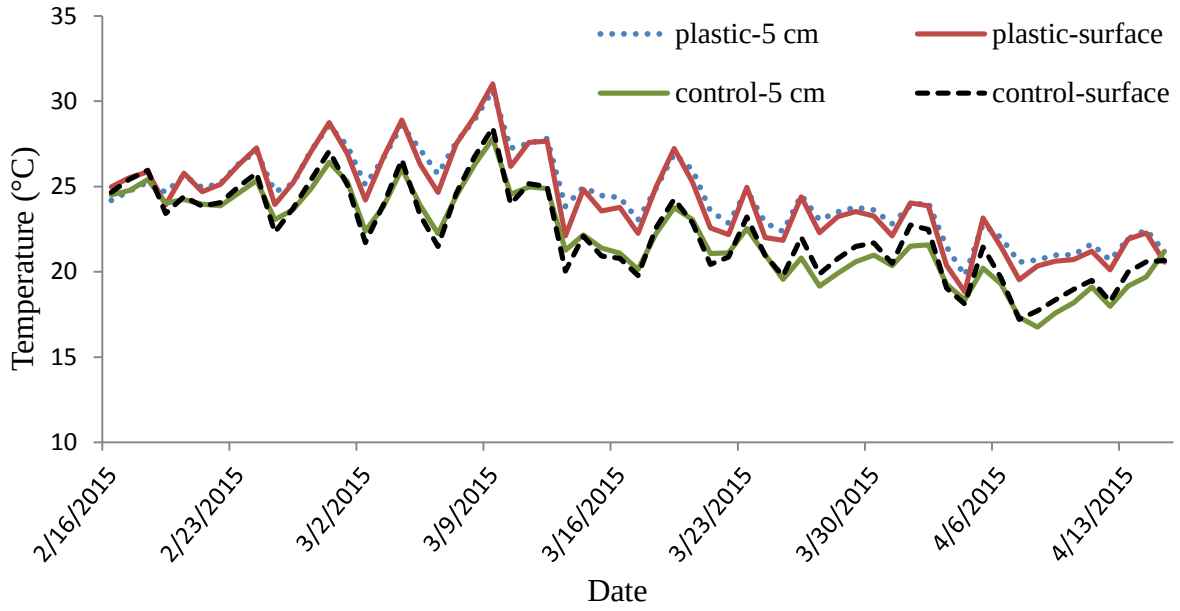


Figure 5.8 The effect of solarisation and non-solarisation (control) treatments on the soil temperature (°C) at the surface and 5 cm depth at Pye Farm, Bringelly. Data points are mean values, n = 4, *P* <0.001.

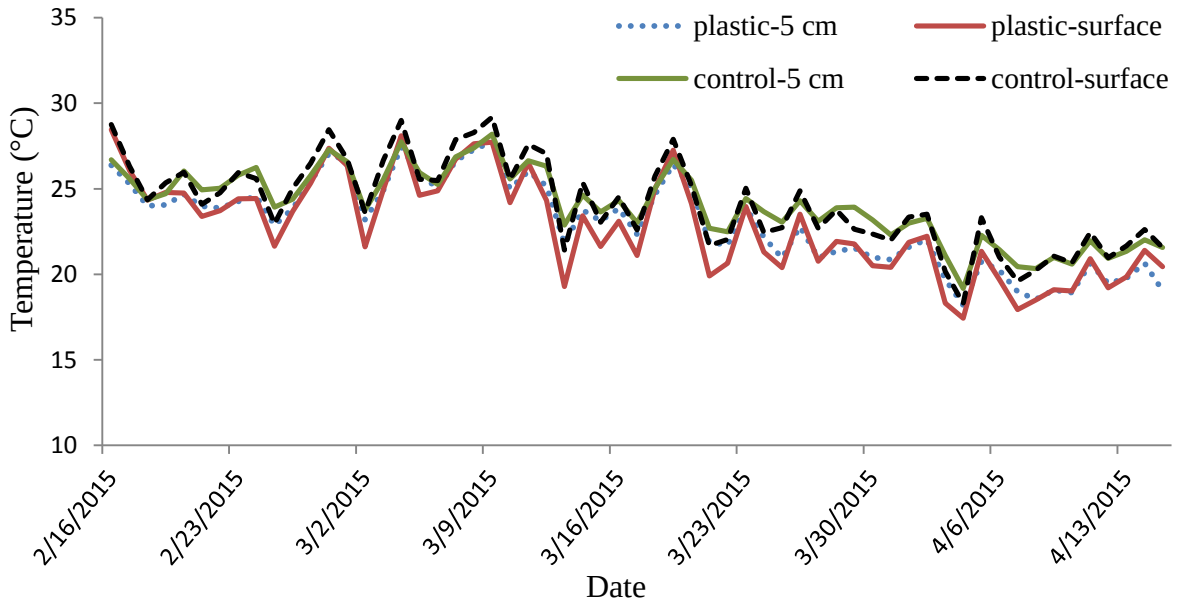


Figure 5.9 The effect of solarisation and non-solarisation (control) treatments on the soil temperature (°C) at the surface and 5 cm depth at Karalee farm, Camden. Data points are mean values, n = 4, *P* <0.001.

Table 5.4 Differences in daily minimum, mean and maximum temperatures (°C) between the solarisation and control treatments throughout the field experiment at the two sites. Values are mean, n = 4. **Highly significant (P <0.001) using paired t-test.

Site	Temperature	Temperature difference (°C)	
		Soil surface	5 cm depth
Karalee Farm	Minimum	2.8**	2.9**
	Maximum	3.6**	4.1**
	Mean	3.3**	3.5**
Pye Farm	Minimum	2.9**	4.3**
	Maximum	0.7**	4.6**
	Mean	2.3**	4.5**

Statistical analysis provided by Dr Kim-Yen Phan-Thien, University of Sydney.

The number of hours where temperatures exceeded 37°C or 40°C was found to be greater in the fallow-control solarisation treatments than the cover crop-solarisation treatments (Tables 5.5–5.8). Solarisation commenced in mid-February (summer) and was carried out for a period of 8 weeks for the fallow-control treatments whereas the cover crop treatment was started in late-March (autumn) and remained in place for about 3 weeks.

Table 5.5 Soil temperatures in fallow-control solarisation plots at Karalee Farm, Camden. The data represents an hourly record of soil temperatures exceeding 37°C or 40°C. Values are mean, n = 4. Plas = black plastic treatment, Cont = control, no plastic treatment, T = temperature.

Time after commencement of solarisation treatment	**Surface temperature**				**Sub-soil (5 cm) temperature**			
	Hours of T ≥37°C		**Hours of T ≥40°C**		**Hours of T ≥37°C**		**Hours of T ≥40°C**	
	Plas	**Cont**	**Plas**	**Cont**	**Plas**	**Cont**	**Plas**	**Cont**
Week 1 (16/02–23/02)	6	4	2	2	0	0	0	0
Week 2 (24/02–2/03)	14	10	7	3	3	0	0	0
Week 3 (03/03–9/03)	33	8	18	2	15	0	4	0
Week 4 (10/03–16/03)	14	0	6	0	2	0	0	0
Duration (17/03–15/04)	14	0	5	0	3	0	0	0
Total	81	22	38	7	23	0	4	0

Data provided by Dr Kim-Yen Phan-Thien, University of Sydney.

Table 5.6 Soil temperatures in fallow-control solarisation plots at Pye Farm, Bringelly. The data represents an hourly record of soil temperatures exceeded 37°C or 40°C. Values are mean, n = 4. Plas = black plastic treatment, Cont = control, no plastic treatment, T = temperature.

Time after commencement of solarisation treatment	**Surface temperature**				**Sub-soil (5 cm) temperature**			
	Hours of T ≥37°C		**Hours of T ≥40°C**		**Hours of T ≥37°C**		**Hours of T ≥40°C**	
	Plas	**Cont**	**Plas**	**Cont**	**Plas**	**Cont**	**Plas**	**Cont**
Week 1 (16/02–23/02)	19	17	11	13	0	0	0	0
Week 2 (24/02–2/03)	13	14	7	8	0	0	0	0
Week 3 (03/03–9/03)	30	18	15	7	0	0	0	0
Week 4 (10/03–16/03)	13	5	4	2	0	0	0	0
Duration (17/03–15/04)	17	21	4	6	0	0	0	0
Total	92	75	41	36	0	0	0	0

Data provided by Dr Kim-Yen Phan-Thien, University of Sydney.

Table 5.7 Soil temperatures in cover crop-solarisation plots at Karalee Farm, Camden. The data represents an hourly record of soil temperatures exceeded 37°C or 40°C. Values are mean, n = 4. Plas = black plastic treatment, Cont = control, no plastic treatment, T = temperature.

Time after commencement of solarisation treatment	Surface temperature				Sub-soil (5 cm) temperature			
	Hours of T ≥37°C		Hours of T ≥40°C		Hours of T ≥37°C		Hours of T ≥40°C	
	Plas	Cont	Plas	Cont	Plas	Cont	Plas	Cont
Week 1 (23/03–30/03)	4	0	0	0	0	0	0	0
Week 2 (31/03–6/04)	1	0	0	0	0	0	0	0
Week 3 (07/04–13/04)	0	0	0	0	0	0	0	0
Total	5	0	0	0	0	0	0	0

Data provided by Dr Kim-Yen Phan-Thien, University of Sydney.

Table 5.8 Soil temperatures in cover crop-solarisation plot at Pye Farm, Bringelly. The data represents an hourly record of soil temperatures exceeded 37°C or 40°C. Values are mean, n = 4. Plas = black plastic treatment, Cont = control, no plastic treatment, T = temperature.

Time after commencement of solarisation treatment	Surface temperature				Sub-soil (5 cm) temperature			
	Hours of T ≥37°C		Hours of T ≥40°C		Hours of T ≥37°C		Hours of T ≥40°C	
	Plas	Cont	Plas	Cont	Plas	Cont	Plas	Cont
Week 1 (23/03–30/03)	6	10	0	1	0	0	0	0
Week 2 (31/03–6/04)	0	0	0	0	0	0	0	0
Week 3 (07/04–13/04)	0	0	0	0	0	0	0	0
Total	6	10	0	1	0	0	0	0

Data provided by Dr Kim-Yen Phan-Thien, University of Sydney.

5.3.4 Survival of *Salmonella* over time

Time had a significant effect on the rate of decline of *Salmonella*. To investigate the decline of *Salmonella* over time regardless of the treatment applied, data only control treatments over the period of 14 weeks were compared. Generally, a decreasing trend in the survival of

Salmonella was observed between days 0–56 except day 63, in which there was a sharp and significant decline in numbers. Between days 70–105 the count declined over time but there was no significant difference among the sampling points. The *Salmonella* count fell below the limit of detection after day 91 (Figure 5.10). The initial inoculum level was 7 $\log_{10}$ CFU g^{-1}.

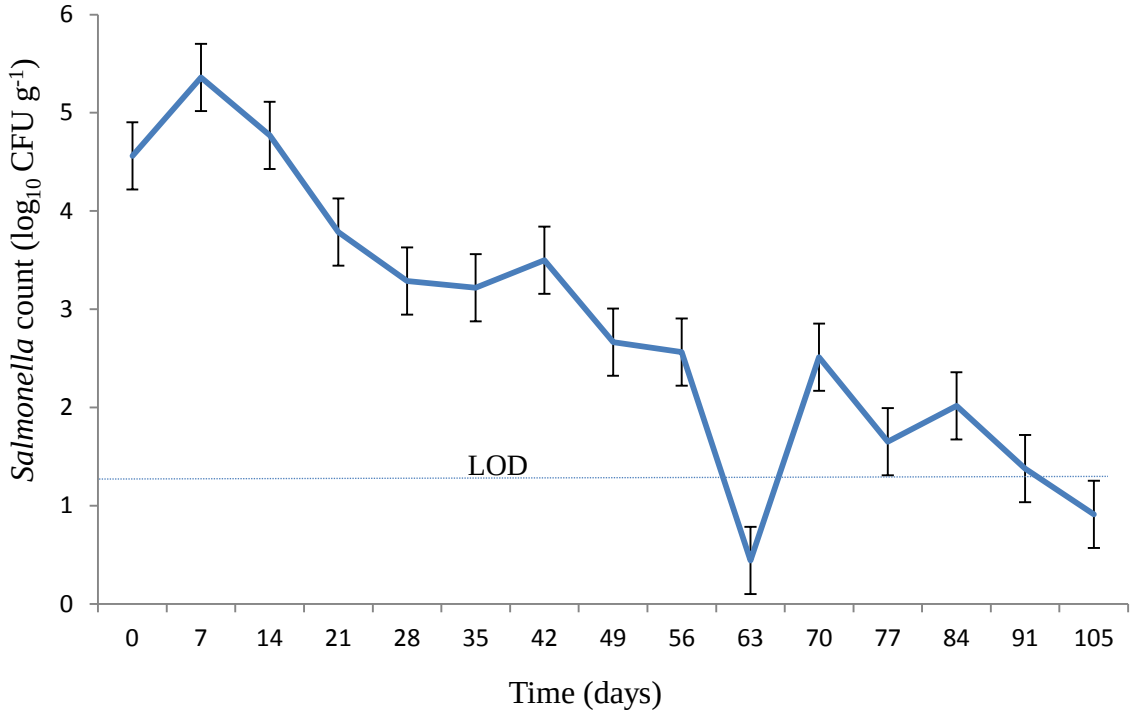


Figure 5.10 *Salmonella* survival in clay soil amended with chicken manure over time under field conditions. Data represents only control treatments. Data points are mean ± standard error, n = 4, P <0.001. The quantitative limit of detection (LOD; 1.18 $\log_{10}$ CFU g^{-1}) of the viable count was determined by direct plating. CFU = colony forming unit.

5.3.5 Survival of *Salmonella* in clay and sandy soils

Between day 7 and day 14 the *Salmonella* count in the sandy soil had dropped by 4 $\log_{10}$ to below the LOD (1.18 $\log_{10}$ CFU g^{-1}) whereas the rate of *Salmonella* decline in clay soil was considerably slower (Figure 5.11) with the count being 3.24 $\log_{10}$ CFU g^{-1} at day 14. Over the 35 day experimental period, *Salmonella* declined by 2.0 $\log_{10}$ CFU g^{-1}.

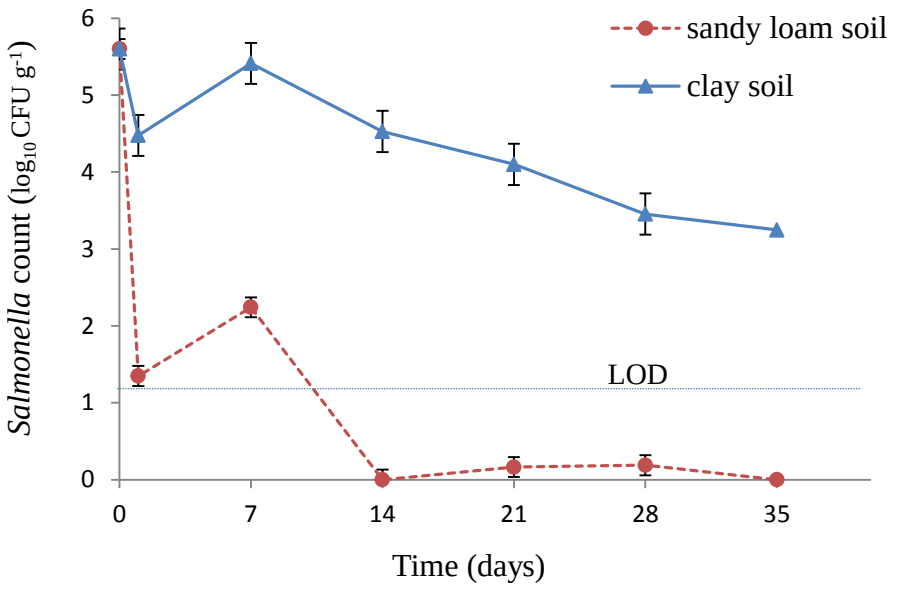


Figure 5.11 The survival rate of *Salmonella* in clay and sandy soils amended with chicken manure. The data represent whole plot sample means ± standard error, n = 16, *P* <0.0001. The quantitative limit of detection (LOD; 1.18 $\log_{10}$ CFU g^{-1}) of the viable count was determined by direct plating. CFU = colony forming unit.

5.3.6 Effect of cover crop treatments on the survival of *Salmonella*

After 4 weeks of growth, the lettuce crop was incorporated into the soil and cover crop treatments were applied. The cover crop treatments (mustard, radish, sorghum and control or fallow) had no significant effect on the survival of *Salmonella* in the soil. The cover crops were sown and incorporated into the soil on day 33 and day 68, respectively of the experiment. The rate of decline in *Salmonella* numbers followed a similar trend in all treatment combinations including the control (fallow) (Figure 5.12).

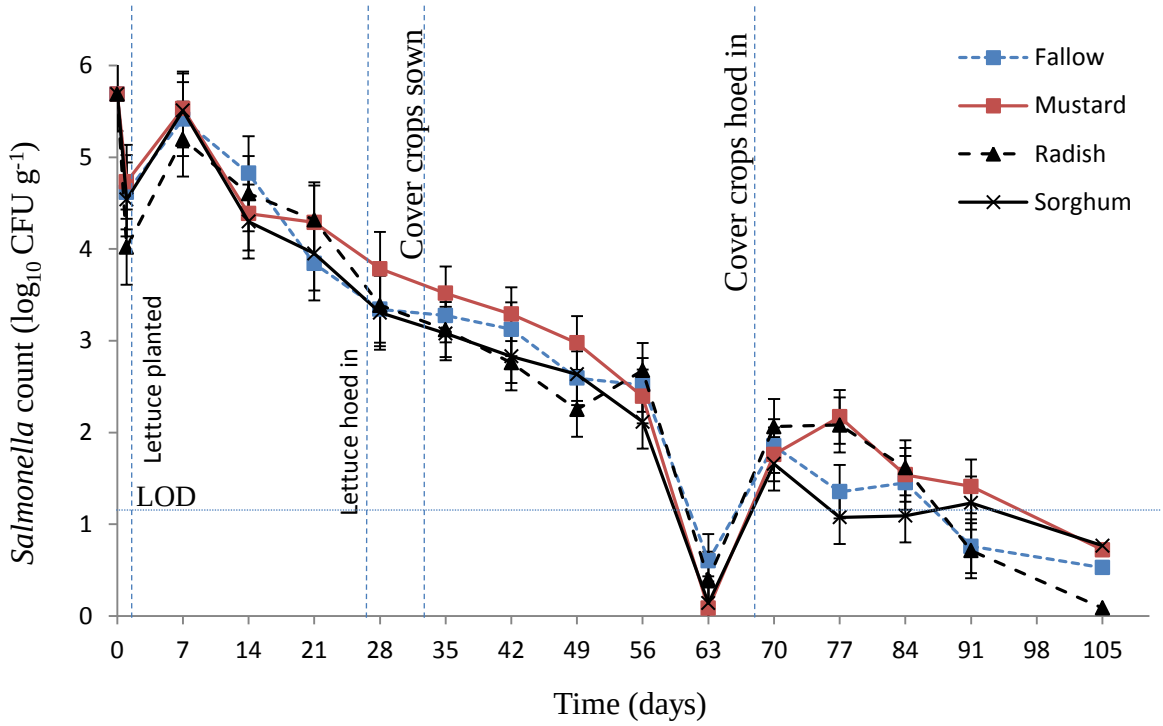


Figure 5.12 *Salmonella* survival in clay soil amended with chicken manure after sowing and growing of cover crops (days 33–67) and ploughing in of cover crops (days 68–105). Day 0–32 are whole plot sample means ± standard error, n = 4, P = 0.587; day 33–105 are split-plot sample means ± standard error, n = 8, P = 0.902. The quantitative limit of detection (LOD; 1.18 $\log_{10}$ CFU g^{-1}) of the viable count was determined by direct plating. CFU = colony forming unit.

The growth of the cover crop was highly variable and sparse in some areas as observed in the overhead photographs of the field plots (data not shown). A crude ground cover index was estimated using image manipulation software (Vectorworks 2015 SP1, distributed by OzCAD in Australia) (Figure 5.13).

When considering the four replicate whole plots for each cover crop grown on sandy soil, the best coverage was by the mustard treatments (63–85%), followed by radish (43–79%), while sorghum resulted in the least coverage (19–58%) (data not shown). For the clay soil site, the best coverage was by mustard (75–87%), followed by sorghum (37–78%) and radish (32–56%). Figure 5.13 depicts the range of cover crop growth at the site with clay soil.

Because of the poor efficacy of the cover crop treatment in the field experiment, the phenolic and glucosinolate compounds were not identified or quantified.

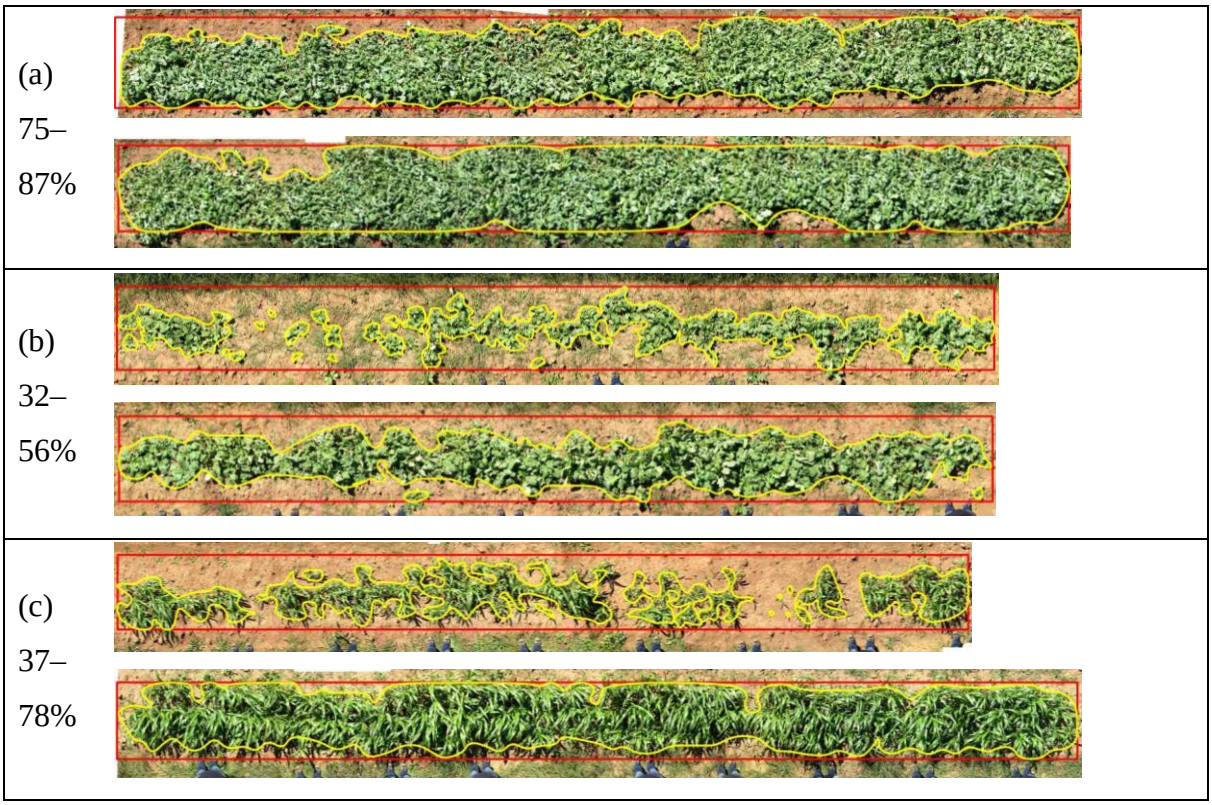


Figure 5.13 Overhead photographs of field plots at Pye Farm, Bringelly (clay soil) demonstrating the variability in groundcover (% estimate) achieved for whole plots of (a) 'Cappuccino' Ethiopian Mustard; (b) 'Terranova' Oilseed Radish; and (c) 'Fumig8tor' Sorghum. Photo provided by Dr Kim-Yen Phan-Thien, University of Sydney.

5.3.7 Effect of soil solarisation treatment on the survival of *Salmonella*

Soil solarisation had a highly significant effect on the decline of *Salmonella* in clay soil (P = 0.0005). All treatments involving solarisation were found to promote the decline of *Salmonella* numbers, however, there was no significant difference among fallow-solarisation, mustard-solarisation and all non-solarised plots (Table 5.9).

Table 5.9 *Salmonella* survival in clay soil with chicken manure amendments with or without solarisation treatment. The count data are the pooled means 20 samples (4 replications and 5 weeks sampling) ± standard error. Mean values followed by the same letter are not significantly different (Tukeys HSD post-hoc test). CFU = colony forming unit; open = non-solarised treatments.

Split plot treatment	***Salmonella* count (log_{10} CFU g^{-1})**
Sorghum-solarisation	0.54 ± 0.22^{a}
Fallow-solarisation	0.56 ± 0.22^{a}
Mustard-solarisation	1.26 ± 0.22^{ab}
Radish-solarisation	1.39 ± 0.26^{ab}
Radish-open	1.43 ± 0.26^{ab}
Mustard-open	1.64 ± 0.22^{b}
Fallow-open	1.68 ± 0.22^{b}
Sorghum-open	1.86 ± 0.22^{b}

The effect of solarisation and control (open) treatments on the survival of *Salmonella* was compared by pooling the data between days 70–105. *Salmonella* exhibited the poorest survival in the sorghum-solarisation treatment plots. Fallow-solarisation was the next least effective treatment for the decline of *Salmonella*, even though it was started 5 weeks earlier than the sorghum-solarisation treatment (Figure 5.14). However, these two treatments (sorghum-solarisation and fallow-solarisation) were not statistically different from the mustard and radish solarisation treatments (Table 5.9).

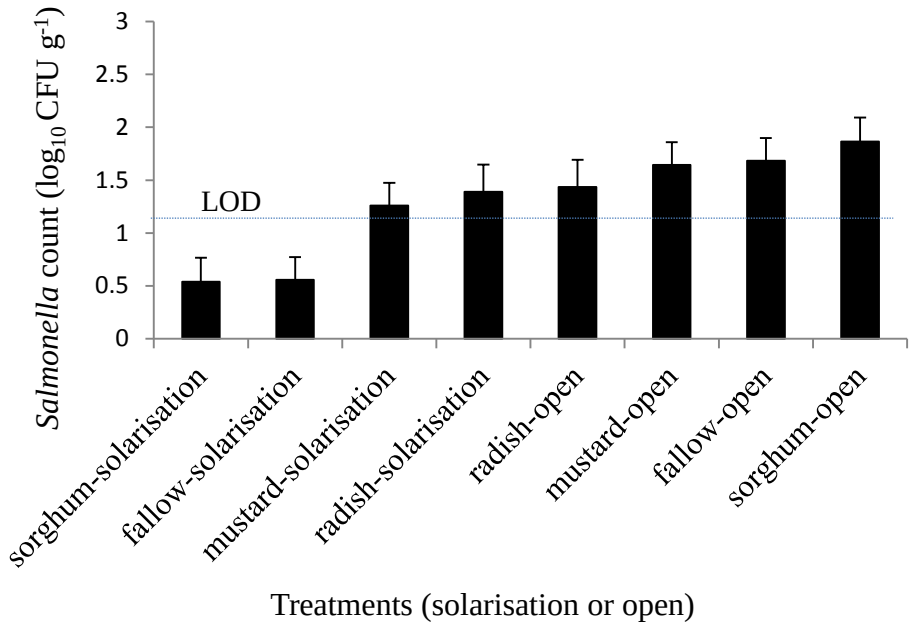


Figure 5.14 *Salmonella* survival in chicken manure-amended clay soil with or without solarisation treatments (between days 70–105). Bars are mean ± standard error, n = 20. The quantitative limit of detection (LOD; 1.18 $\log_{10}$ CFU g^{-1}) of the viable count was determined by direct plating. CFU = colony forming unit.

The presence or absence of residual viable *Salmonella* cells was detected by following enrichment steps described in the methods (see Chapter 5.2.14). The presence/absence data was not statistically analysed because most of the results obtained after enrichment were found to be positive and few were negative (Figure 5.15). Only two examples are presented to show the proportion of samples that could be enumerated or detected by enrichment (below the LOD). On sampling day 105, in sorghum-solarisation treatment soils (Figure 5.15a), 100% of the samples were enriched and found to be positive for *Salmonella* cells, whereas, in sorghum-open treatment soils (Figure 5.15b), 75% of the samples were directly counted and the remaining 25% of the samples were enriched and all were positive.

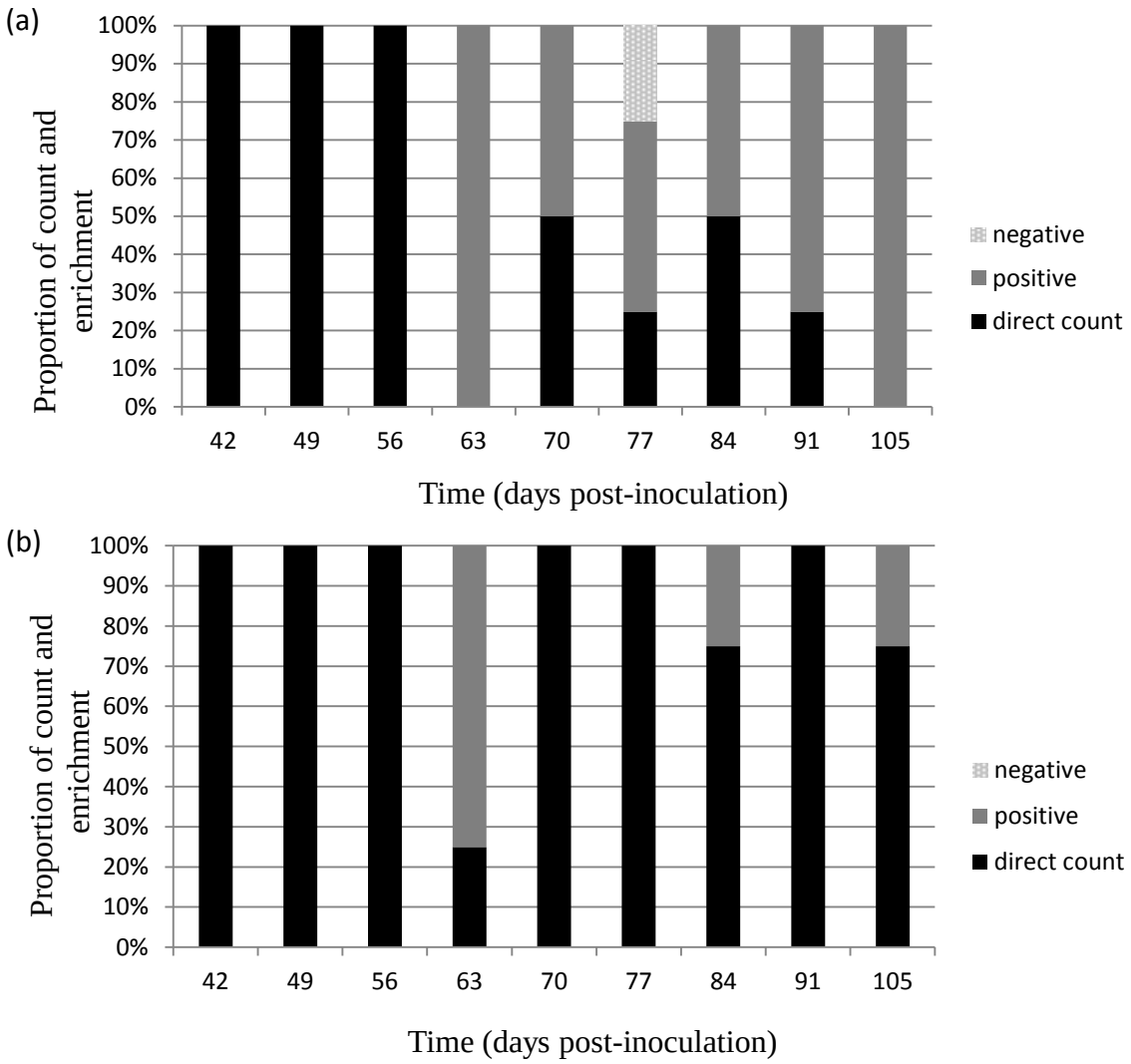


Figure 5.15 The proportion of *Salmonella* cells recovered using direct plating and enrichment (positive or negative) from (a) sorghum-solarisation and (b) sorghum-open treatments at Pye Farm, Bringelly (clay soil).

In the same pattern, on sampling day 105, in fallow-solarisation treatment soils (Figure 5.16a), 100% of the samples were enriched and found to be positive for *Salmonella* cells, whereas, in fallow-open treatment soil (Figure 5.16b), 50% of the samples were directly counted and the remaining 50% of the samples were enriched and all were positive. The remaining graphs for each treatment (solarisation or open) are provided in Appendix 4.

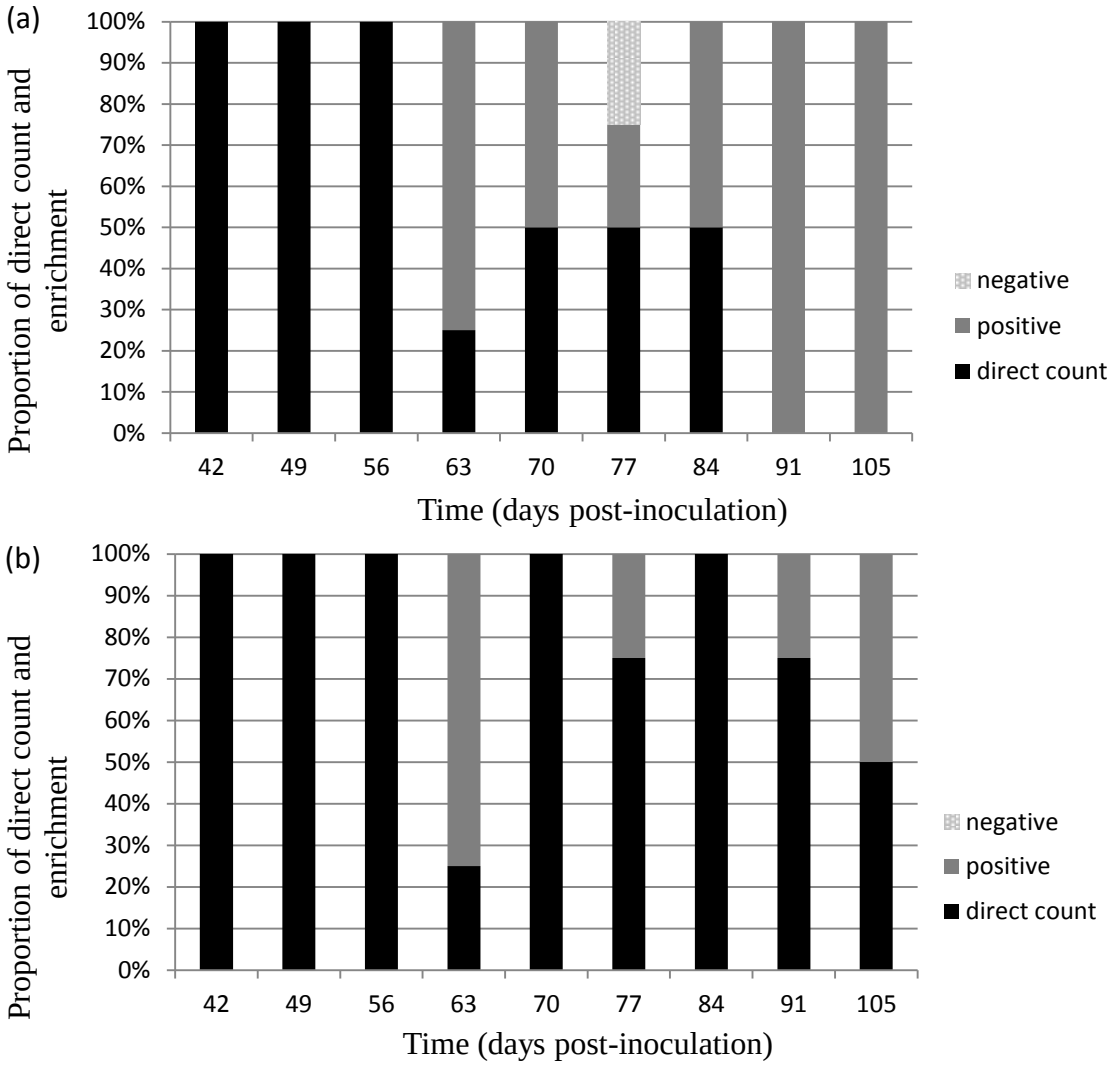


Figure 5.16 The proportion of *Salmonella* cells recovered using direct plating and enrichment (positive or negative) from (a) fallow-solarisation, (b) fallow-open treatments at Pye farm, Bringelly (clay soil).

In the first 28 days under the control treatment in the clay soil (since there was no treatment at this stage of the experiment), the reduction of *Salmonella* cells between day 7 and day 28 was 40% (Figure 5.17). This coincides with hot summer daily temperatures between 30 to 40°C. Afterwards, the rate of decline in *Salmonella* numbers was slower except at day 63 where an abrupt decrease in cell counts was observed (Figure 5.17). The solarisation treatment on day 35 promoted the decline of *Salmonella* until day 56 when levels fell below the LOD (Figure 5.17). In contrast, *Salmonella* was detected in the control treatment up to day 91 post inoculation.

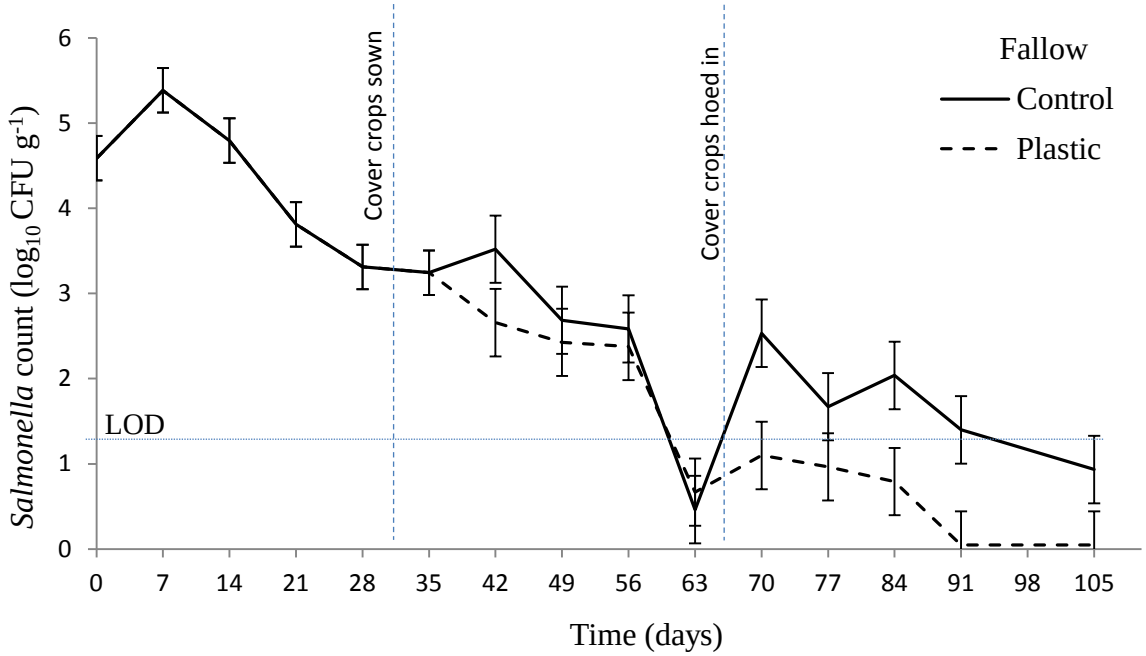


Figure 5.17 *Salmonella* survival in chicken manure-amended clay soil with or without (fallow) solarisation treatments between days 33–105. Data points are mean ± standard error, n = 4. The quantitative limit of detection (LOD; 1.18 $\log_{10}$ CFU g^{-1}) of the viable count was determined by direct plating. CFU = colony forming unit.

The sorghum-solarisation treatment was found to be the better in hastening the rate of decline in *Salmonella* survival and there was a highly significant (P = 0.0013) difference between the sorghum-solarisation and sorghum control plots (Figure 5.18). After the incorporation of the sorghum cover crop and the solarisation treatment, *Salmonella* counts were below the LOD in solarised plots, whereas *Salmonella* was detected up to 105 days post-inoculation in non-solarised plots. At the last sampling point (day 105), a count of 1.64 $\log_{10}$ CFU g^{-1} was recorded in the sorghum control treatment, whereas the count from the sorghum-solarisation treatment plot was below the LOD.

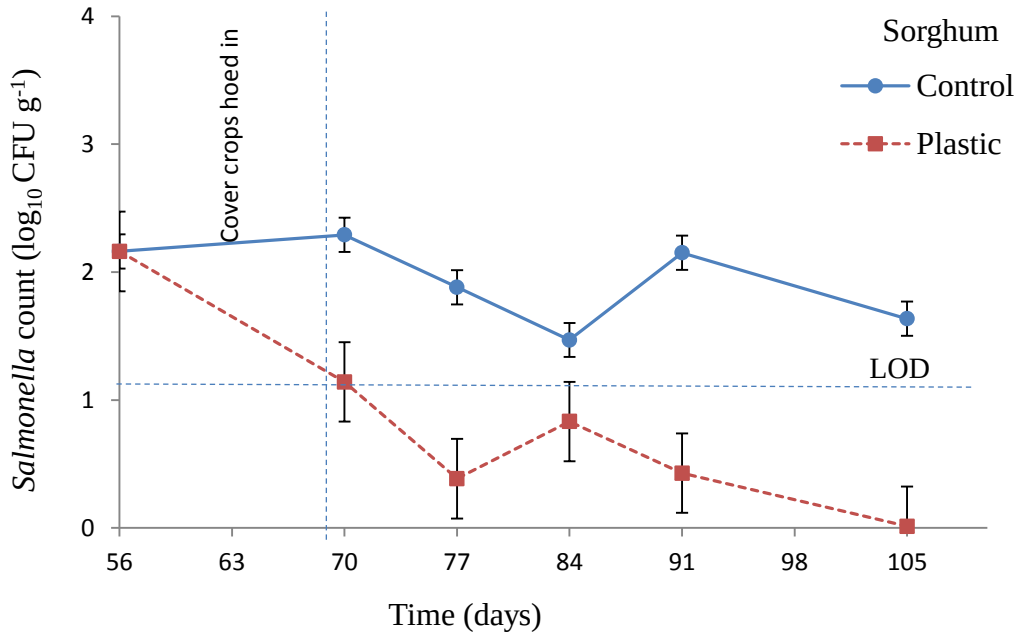


Figure 5.18 *Salmonella* survival in chicken manure-amended clay soil between 56–105. The treatment included sowing and growing 'Fumig8tor' sorghum cover crop (day 33–67), cover crop incorporation (day 68–105) and either left fallow (control) or solarisation treatment. Data points are mean ± standard error, n = 4, P <0.001. The quantitative limit of detection (LOD; 1.18 $\log_{10}$ CFU g^{-1}) of the viable count was determined by direct plating. CFU = colony forming unit.

5.3.8 *Listeria monocytogenes* population during the field experiment

The presence/absence of *L. monocytogenes* was monitored throughout the field experiment, by enriching and culturing soil samples that were collected and pooled in the same manner as for the *Salmonella* analysis. Viable *L. monocytogenes* was detected in all of the samples tested for all of the treatments (data not shown). This is to be expected as *Listeria* is a common soil microorganism. As there was no difference between treatments, no statistical analysis on this data was performed.

5.4 Discussion

In the present study, *Salmonella*-inoculated chicken manure was applied to sandy and clay soils. The decline of *Salmonella* cells due to soil biofumigation and/or solarisation over time was evaluated. The effects of the different treatments used to suppress the survival of *Salmonella* in the study will be discussed below in relation to the effects of soil type, soil and weather conditions.

5.4.1 Soil type effects on *Salmonella* survival

Time had a significant effect on the decline of *Salmonella* in clay soil. There was a 3.0 log decline over 105 days compared to a similar decline which occurred over just 14 days in the sandy soil. At day 14 the level of *Salmonella* cells detected in sandy was below the LOD, whereas it was still 1.42 $\log_{10}$ CFU g^{-1} (i.e. above the LOD) in clay soil on day 84. The long term survival of *Salmonella* in clay soil during the field experiment was similar to the results obtained for the pot experiment in Chapter 4. In the laboratory experiment the same soil types were used as for the field trails and the experiment conducted under controlled conditions within the temperature and moisture ranges found in the field. In the field experiment environmental variables were not controlled however the survival of *Salmonella* in both instances were similar. These results are also in agreement with previous research which reported longer term survival of *Salmonella* in clay soil than in sandy or sandy loam soils (van Veen *et al.* 1997; Danyluk *et al.* 2008; Franz and van Bruggen 2008).

Jamieson *et al.* (2002) reported that the mortality of bacterial pathogens is influenced by soil type, with soils that have higher matric potential promoting lower microbial mortality rates. Matric potential is defined as adhesion of water molecules to non-dissolved structures of the system. Some of the major soil factors that contribute to the difference in survival of *Salmonella* in the two soil types (sandy and clay) are described in Chapter 4. In addition, Brennan *et al.* (2014) reported that an increased surface area, resulting from a smaller average particle size in clay soil, will result in the protection of bacterial pathogens from predators, desiccation, UV radiation and toxins. Any or all of these factors may have contributed to the survival of *Salmonella* cells for more than 100 days in the clay soil used in this study. In addition, different types of clay size fractions consist of different clay mineral types with each having its own specific physicochemical and mineralogical properties (i.e. particle size, surface area, shape, cation exchange capacity, moisture absorption, elasticity and provision of

mineral nutrients) that may affect bacterial pathogen survival (England *et al.* 1993; Höper *et al.* 1995).

5.4.2 Effect of soil moisture on *Salmonella* survival

Previous studies indicate the significant contribution of soil moisture to the survival of bacterial pathogens in soil or manure-amended soil (Chandler and Craven 1980; Hayes *et al.* 2000; Lang and Smith 2007; Aislabie *et al.* 2011; Kim *et al.* 2012). Lang and Smith (2007) reported that the decline of enteric organisms is influenced by soil and environmental factors, including soil texture, soil organic matter, pH values, temperature and moisture content. Soil moisture is particularly identified as a principal factor affecting pathogen survival in the field. In the field, soil moisture content fluctuated with rainfall events and irrigation treatments as they would be expected to in a commercial setting. Generally, the soil moisture was maintained in the range of 10–20% in the sandy soil and 20–45% in the clay soil. However, soil moisture at the clay site decreased to below 20% water by volume when sampled on days 56 and 63. It is possible that the observed die-off of *Salmonella* followed a typical trajectory or that the die-off was expedited by the unintended dry spell, although many factors contributed to die-off. In either case, there was increased recovery of *Salmonella* on day 70. This coincided with the incorporation of cover crops, accompanied by irrigation. As there were several confounding factors that may explain the change in microbial population, it is not possible to make conclusions about their relative importance. However, in the pot experiment (Chapter 4), the survival of *Salmonella* was found to be better under constant moisture regimes rather than fluctuating moisture in both soil types, with or without chicken manure amendments. As such, further research would be worthwhile to better understand the role and interactions of irrigation/rainfall, temperature and agronomic treatments to control *Salmonella* population in a field production context.

5.4.3 Biofumigation effects on *Salmonella* survival

The *Salmonella* population in the sandy soil declined to below the LOD prior to the application of agronomic treatments (Figure 5.18). Hence, any further discussion of the different treatments and their effects on the pathogen will focus on the results obtained with the clay soil.

None of the cover crop treatments (Ethiopian mustard, oilseed radish and sorghum) had a significant effect on the *Salmonella* population of the chicken manure-amended soil when

compared with the control (fallow) treatment. It was reported that the glucosinolate hydrolysis products, in particular ITCs, are known to have broad biocidal activity (Brown and Morra 1997; Kirkegaard *et al.* 1997; Rosa *et al.* 1997). The ineffectiveness of the cover crops used in the present study may have been due to inadequate growth of the cover crop, leading to low levels of biomass being incorporated into the soil, and insufficient release of biofumigant compounds. According to Bellostas *et al.* (2004), optimal biofumigation, requires a high production of glucosinolate resulting from high dry matter production towards the end of the growing period of cruciferous plants. However, many other factors affect the outcome of biofumigation such as temperature and microbiome for example. The timing of the incorporation of the cover crop may be another factor that limited any biofumigation effects. The cover crops were incorporated into the soil only 4 weeks after sowing in order to expedite replanting of the lettuce crop, which would be desirable for a commercial vegetable growing scenario. According to Kruger *et al.* (2013), the flowering stage of the plant results in higher glucosinolate content than the vegetative growth stages. In addition, Bellostas *et al.* (2004) indicated that the growth stage of the crop (emergence, rosette, flowering, seed filling, ripening), the amount of biomass produced, and the correct incorporation into the soil all contribute towards the success of biofumigation.

Plant age and morphology can be associated with glucosinolate content. Rosa *et al.* (1997) reported that in *Brassica napus* and *B. campestris*, the aliphatic glucosinolates showed relatively high concentrations at the beginning of the vegetative period, however concentrations decreased throughout vegetative development. In the floral parts, concentrations increased. In addition, indole glucosinolate content of kale was significantly higher in plants with thin stems, whereas plants with thick stems were higher in glucosinolates, isothiocyanates and oxazolidinethiones (Rosa *et al.* (1997).

Different factors affect the production and degradation of glucosinolates which also influence the outcome of biofumigation. For instance, winter and autumn induce lower glucosinolate levels due to short days, wetter conditions, cool temperatures and less radiation (Rosa *et al.* 1997). In the present study, the cover crop was grown and incorporated in autumn which might partly contribute for the ineffectiveness of the cover crops. Water is also another factor affecting the level of glucosinolates and as well as their degradation process. For example, the concentration of glucosinolate was higher in oilseed rape which was exposed to periods of drought at pre- or post-flowering stages (Rosa *et al.* (1997). In addition, sugars and amino

acids in Brassicas that accumulated when the crop was grown under poor conditions were differentially converted to secondary metabolites such as glucosinolates, rather than cellulose and proteins (Rosa *et al.* 1997). Brassicas require significantly more sulfur than most other crops due the synthesis of glucosinolates and sulfur amino acids and proteins. As a result, application of sulfur was shown to increase the glucosinolate content of *B. napus* (Booth *et al.* 1991; Rosa *et al.* 1997). Although nitrogen is a constituent of glucosinolate molecules, early studies showed that increasing application of nitrogen led to lower glucosinolate levels. Boron nutrition was also found to play a significant role in the regulation of glucosinolate biosynthesis while copper cations in plant tissues has been linked to a decrease in glucosinolate content (Rosa *et al.* 1997).

5.4.4 Effect of solarisation treatments on *Salmonella* survival

In this study, the combined effect of soil solarisation and biofumigation treatments were not significant in suppressing *Salmonella* survival in chicken manure-amended soil. However, Gamliel and Stapleton (1993) reported that the microbial activity in heated soil amended with cabbage was reduced rapidly during the first week of incubation at 38°C, when compared with heated, non-amended soil. This was due to the combined effects of the heat and the toxicity of the volatile compounds from cabbage. In the course of this study, the solarisation treatment was applied after summer (in March) and remained for only approximately 3 weeks; this might be the reason for the ineffectiveness of the soil solarisation-cover-crop treatments.

In contrast, the soil solarisation treatment alone was found to be significant in reducing the survival of *Salmonella* in the soil. Temperatures of more than 37°C were recorded for 92 h and 75 h, respectively, for the fallow-solarisation and fallow-control treatments over the 4 week soil solarisation treatment. Similarly, temperatures greater than 40°C were recorded for 41 h and 36 h, respectively for fallow-solarisation and fallow-control treatments during the solarisation period (Tables 5.5–5.8). The greater reduction in *Salmonella* populations in solarised soil compared with non-solarised soil may well be attributed to the higher temperature reached and sustained. In support of this hypothesis, it has been reported that soil solarisation during summer in the southern part of Japan was effective in raising the soil temperature to more than 40°C resulting in the rapid inactivation of *E. coli* in an open upland field (Wu *et al.* 2009). It has also been reported that the temperature of soil amended with compost or manure increased by 2–3°C, compared with solarised but non-amended soil

possibly due to increased soil moisture and thermal conductivity in the compost-amended soil and exothermic microbial activity (Gamliel and Stapleton 1997).

5.4.5 Prevalence of *Listeria monocytogenes* in field soils

The purpose of detecting *L. monocytogenes* in the present study was to observe the potential increase in bacteria particularly after incorporation of cover crops since *L. monocytogenes* is commonly found in plants undergoing decay (Fenlon 1999). However, there was no difference in the detection of *L. monocytogenes* in the soil before and after incorporation of the cover crops. According to Vivant *et al.* (2013), *L. monocytogenes* is a telluric bacterium commonly found in soil, water and associated with plants and can survive under adverse environmental conditions longer than many other non-spore-forming bacteria that are important causes of foodborne disease. This resistance, together with the ability to colonise, multiply and persist on processing equipment makes *L. monocytogenes* a particular threat to the food industry (Fenlon 1999). In support of this, the bacteria was detected throughout the experiment without being artificially inoculated (data not shown). The persistent nature of *Listeria* in the soil becomes an important factor to consider for the risk of transmission of the pathogen from contaminated produce to humans.

5.4.6 Microbial community analysis

The microbial community analysis was performed on the field sites as a part of a larger scale project and the results are presented in Gonzalez (2015). Microbial community analysis indicated that soil texture played a significant role in altering the bacterial and fungal communities, and that clay soil had a more consistent microbial composition than the sandy soil. In addition, the cover crop treatments led to greater changes in the microbial community in the sandy soil compared to the clay soil. Radish and mustard cover crop treatments affected the soil microbial community abundance which might be due to a combination of the effects of chemical and biological characteristics typical of the Brassica plants. Furthermore, neither solarisation nor solarisation-cover crop treatments had a significant effect on soil microbial community (Gonzalez *et al.* 2015).

5.4.7 Summary

The results presented in both Chapter 4 and 5 confirm the short-term survival of *Salmonella* in sandy soil either with or without manure amendments. The level of *Salmonella* present in

sandy soil does not pose a food safety hazard but the level in clay soil, particularly under circumstances represented by the fallow-control treatment which remained above the LOD for more than 90 days, may present a risk for produce contamination. Because of the potential for long-term survival of *Salmonella* in clay soil or manure-amended clay soil, single or combined cover crop-solarisation combinations were used in efforts to supress the pathogen in the soil. The soil solaristion process was effective in hastening the decline of *Salmonella* in the soil, however the cover crops or cover crop-solarisation treatments were not significantly effective in reducing *Salmonella* survival.

6. General discussion and conclusions

In recent years, a number of foodborne disease outbreaks have been associated with the consumption of vegetables, specifically, leafy greens. Contamination of lettuce and other leafy green produce may occur either in preharvest (Islam *et al.* 2004c) or postharvest stages (Sánchez *et al.* 2012); however, preharvest contamination of fresh produce is of the greatest concern. Animal manure is commonly used as an organic fertiliser in both organic and conventional production systems and it is thought to be the principal source of preharvest contaminanation (Franz and van Bruggen 2008; Semenov 2008). In Australia, poultry litter and raw manure from broiler chicken and egg growers is widely applied to commercial vegetable production sites as a soil amendment and fertiliser.

Most of the postharvest sanitation methods are not effective for leafy greens and are not allowable in organic production system. Postharvest sanitation also removes some of the native microflora which in turn has the potential to decrease competitive pressure on any pathogenic bacteria that may present on produce. Therefore, prevention of preharvest contamination should be the main focus when developing intervention strategies (Franz and van Bruggen 2008).

For the microbiological safety of fresh produce, a thorough understanding of human pathogens in manure, soil and crops, their interaction with other microorganisms and risk factors is required (Franz and van Bruggen 2008). The information obtained from this study has increased our understanding about the behaviour of *Salmonella* and will assist in the development of policies or the modification of existing policies to prevent the spread and cycling of pathogens in the environment in which fresh produce is grown (Table 6.1). Investigating the different environmental factors that affect the survival of bacterial pathogens is essential to understand the preharvest growth of foodborne pathogens.

Table 6.1 Summary of key objectives and findings of this study.

Chapter	Key objectives	Key findings and outcomes	Future directions
2. Literature review	1. Document the incidence of foodborne illness in relation to fresh produce, particularly leafy green vegetables 2. Review how soil and environmental factors affect the survival of *Salmonella* 3. Review the effect of biofumigant cover crops and solarisation on the survival of *Salmonella* and other pathogenic soil microorganisms	1. Many of the disease outbreaks related to contamination of fresh produce by pathogenic microorganism have been due to *Salmonella* and *Listeria monocytogenes* 2. A number of biotic and abiotic factors can affect the survival of microorganisms but the evidence for strong patterns is not always evident 3. The effect of biofumigant cover crops and solarisation on *Salmonella* is conflicting and evidence for an enhanced effect when applied in combination is non-existent	Further investigations using controlled conditions are needed to determine the effects of environmental factors on *Salmonella* survival in soil Test the efficacy of cover crop biofumigation and solarisation for remediation of soil contaminated with *Salmonella*
4. Microcosm pot trial	1. To investigate the effect of environmental factors (e.g. moisture, temperature, soil type) on the survival of *Salmonella* serovars 2. To interpret the survival pattern of *Salmonella* serovars for application of findings in a field experiment	1. Faster die-off of *Salmonella* in sandy soil compared to clay soil 2. Decline of *Salmonella* was hastened when high temperature was coupled with fluctuating moisture regime 3. A longer period of time between the application of fresh or partially composted manure and planting of vegetables is required for clay soil	Soil characteristics should be tested in relation to die-off of *Salmonella* Assess the relative importance of environmental factors for growth among *Salmonella* serovars
5. Field experiment	1. Determine the optimum low-residue cover crop to enhance die-off of *Salmonella* in contrasting soil types 2. Evaluate the best treatment for facilitating die-off of *Salmonella* (cover crop, solarisation or cover crop-solarisation) 3. Assess to presence of *L. monocytogenes* in the field	1. The survival of *Salmonella* in clay soil was enhanced when amended with chicken manure 2. Solarisation was effective in enhancing die-off of *Salmonella* in the soil 3. Cover crop or cover crop-solarisation treatments were not effective in enhancing die-off of *Salmonella* in the soil	Cover crop coverage, plant developmental stage and incorporation methods need to be further assessed to determine biofumigation potential Biocidal compounds of biofumigant cover crops should be identified and quantified More field studies on *Salmonella* decline should be done in different seasons, regions, climates and soil types in Australia How biotic control *Salmonella* decline in soil should be investigated

Environmental factors

Variation in the duration of the survival period of bacteria is attributed to various environmental factors such as the physical and chemical properties of the soil, temperature and moisture (van Veen *et al.* 1997). Therefore, the first focus of this study (Chapter 4) was to identify factors and interactions that may minimise *Salmonella* survival in soil or chicken manure-amended soil. A study using microcosm pots using two different soil types and bacterial inocula demonstrated that clay soil supported the survival of *Salmonella* serovars more than sandy soil. With regard to soil type, the results of the microcosm pot trial were confirmed in the field experiment (Chapter 5), where *Salmonella* counts in the sandy soil had dropped to below the LOD (1.18 $\log_{10}$ CFU g^{-1}, estimates) after 14 days post-inoculation, whereas the level of *Salmonella* detected in clay soil was still 1.42 $\log_{10}$ CFU g^{-1} on day 84. This finding is supported by the results of Holley *et al.* (2006) who found that *Salmonella* survival was generally longer in heavier soils with high moisture and cooler temperatures. The possible reasons for the enhanced survival of bacterial pathogens in clay soil are an increased surface area due to smaller soil particle size, which protects bacterial pathogens from predators, UV radiation and toxins (Brennan *et al.* 2014). The longer survival time of *Salmonella* in clay soil could lead to product contamination through rain splash (Cevallos-Cevallos *et al.* 2012a; Cevallos-Cevallos *et al.* 2012b; Gu *et al.* 2013) or internalisation (Guo *et al.* 2002a; Klerks *et al.* 2007a; Gu *et al.* 2013) poses a higher risk than when grown produce is grown in sandy soils.

Other soil properties that affect the survival of bacterial pathogens are pH and electrical conductivity (EC). In the present study, the relationship of *Salmonella* survival with both EC and pH was found to be inconsistent. Erickson *et al.* (2014) reported that the influence of pH on the persistence of bacterial pathogens may be reliant on factors such as nutrient availability and the activity of other biota however, the impact of pH on *E. coli* 0157:H7 survival was unclear. However, pH was found to be a major factor affecting the survival of *E. coli* 0157:H7 in another study (Ma *et al.* 2013). For EC, a negative correlation was reported for the survival of *E. coli* 0157:H7 (Erickson *et al.* 2014).

Soil treated with manure amendments was found to improve the survival of *Salmonella* in the present study. *Salmonella* counts were reduced, on average, by 20% in manure-treated samples whereas the reduction was close to 50% in samples without manure. Chicken manure is a source of contamination in the field and also provides nutrients for the growth and survival of

bacterial pathogens. The addition of manure increases the organic matter content of soil (Santamaria and Toranzos 2003; Franz and van Bruggen 2008), which in turn increases the retention of other nutrients (Jamieson *et al.* 2002), provides protective effects (Dowe *et al.* 1997; Cools *et al.* 2001; Holley *et al.* 2006) and eventually leads to cell repair (Holley *et al.* 2006). This effect was observed particularly in the microcosm pot trial where greater numbers of *Salmonella* were recovered (either by direct plating or by detection (presence/absence) methods) from manure-amended soils when compared with non-manure amended soils.

The other main environmental factor considered in this study was temperature. As detailed in Chapter 4, the survival of *Salmonella* at lower temperature (5°C) was greater than survival at higher temperature (37°C). Similarly, Holley *et al.* (2006) reported that survival of *Salmonella* was higher at 4°C than 25°C. Generally, temperature has a profound effect on the growth and decay rates of bacteria (Ongeng *et al.* 2015), and survival rates of *Salmonella* spp. decline as temperatures increase from 20 to 70°C in natural substrates (Kudva *et al.* 1998; Himathongkham *et al.* 1999). However, the adaptation or resistance of *Salmonella* to lower or higher temperatures is strain-dependent. It was found that the combination of high temperature and fluctuating moisture hastened the decline of *Salmonella* in the soil, with or without manure amendments (Chapter 4). Similarly, Semenov *et al*. (2007) showed that increased temperatures cause greater decline of both *Salmonella* and *E. coli* under a fluctuating temperature regime compared to constant temperature.

In general, a decrease in soil moisture has a direct effect on pathogen decay due to desiccation and autolysis of bacterial cells (Lang and Smith 2007). In the field experiment (Chapter 5), the decline in *Salmonella* leading up to day 63 was considered to be a valid result and may have been exacerbated due to declining soil moisture. The subsequent rise in *Salmonella* counts on the next sampling date occurred immediately after turning in of the cover crop and was followed by irrigation to hasten decomposition of the vegetation. An increase in soil moisture was most likely the reason for increased counts. Similarly, in the microcosm pot trial (Chapter 4), *Salmonella* survived better in constant moisture than in fluctuating moisture regime. According to Lang and Smith (2007), soil moisture is a major factor influencing the survival of pathogens in the field. However, due to confounding factors, particularly the incorporation of cover crops (control plots were also ploughed) and irrigation, it was not possible to make conclusions about the impact of moisture in this field

experiment. Further studies could help to untangle the relative importance of these environmental factors.

Remediation of contaminated soil

Because of the long-term survival of *Salmonella* in soil or manure-amended soil, the current project investigated practices that could be used for the remediation and recovery of soil contaminated by *Salmonella* following the intentional application of chicken manure as a pre-planting fertility management strategy. Single or combined cover crop-solarisation combinations were used in efforts to supress the pathogen in the soil (Table 6.1).

The soil solarisation process was effective in hastening the decline of *Salmonella* in the soil that was left fallow. Soil temperatures greater than 37°C were recorded for extended periods for both the fallow-solarisation and fallow-control treatments over a 4-week treatment period (92 h and 75 h, respectively), and greater than 40°C (41 h and 36 h, respectively) for the same period. The heating effect of the black plastic covering was enough that, on day 70, the average *Salmonella* count from the fallow-control treatment was 2.50 $\log_{10}$ CFU g^{-1} whereas, counts from the fallow-solarised treatments was below the LOD. A greater reduction in the *Salmonella* population in the solarised soil compared with the non-solarised soil may be attributed to the higher temperature in the solarised treatment plots.

In contrast, the cover crop-solarisation treatments were not effective in significantly reducing *Salmonella* survival. The number of hours where temperatures exceeded 37°C or 40°C was found to be greater in the fallow-control solarisation treatments than the cover crop-solarisation treatments. Solarisation was started in mid-February (summer) and was applied out for a period of 8 weeks for the fallow-control treatments whereas the cover crop treatment was started in late-March (autumn) and was applied for only 3 weeks. Increasing the duration of the cover crop-solarisation treatment, as well as the timing of its application to match the fallow-solarisation treatments is advised before disregarding this as a possible strategy to reduce *Salmonella* survival.

Even though glucosinolates are generally considered toxic (Wathelet *et al.* 2004; Gimsing *et al.* 2005) and phenolics possess a biofumigation effect against bacterial pathogens (Cetin-Karaca 2011), the biofumigant cover crop treatments did not have a significant suppression effect on *Salmonella*. The lack of any antimicrobial effect due to the cover crops used in the

present study might be attributed to the sparse growth of the cover crop which may have resulted in inadequate levels of biomass being incorporated into the soil, and hence an insufficient biofumigation effect. The immaturity of the cover crops at the time of incorporation may be another factor that limited the biofumigation effect. The cover crops were incorporated into the soil only 4 weeks after sowing in order to expedite replanting of the lettuce crop. While this did not allow for full growth of the cover crop during the field experiment it is representative of the timeframes used in commercial vegetable growing scenarios.

Factors that could be directly influenced in this study, including the growth stage of the crop (i.e. emergence, rosette, flowering, seed filling, ripening), the amount of biomass produced (production time), and the method of incorporation into the soil (i.e. ploughed in, rolled and irrigated), all contribute towards the success of biofumigation (Bellostas *et al.* (2004) Figure 6.1). The efficacy of glucosinolates as a biofumigation technique also depends on various soil factors (Kruger *et al.* 2013). Other factors that cannot be directly manipulated include tissue breakdown and release of the biofumigants, side-effects on beneficial microorganisms, and interactions with other bioactive compounds present in the plant tissue (Bellostas *et al.* 2004). In the field study, the aim was to simulate expediting return of the field to full production after contamination with *Salmonella.* Therefore, the cover crops were only grown for one month and may not have accumulated sufficient biofumigant activity (biomass: quantity and growth stage, plant tissue, glucosinolate: quality and type; Figure 6.1). The incorporation technique was standard and common soil types were used (clay and sandy soils). As a consequence, we were unable to determine the types and level of biofumigant in the cover crops themselves as well and the soil. This clearly warrants further investigation.

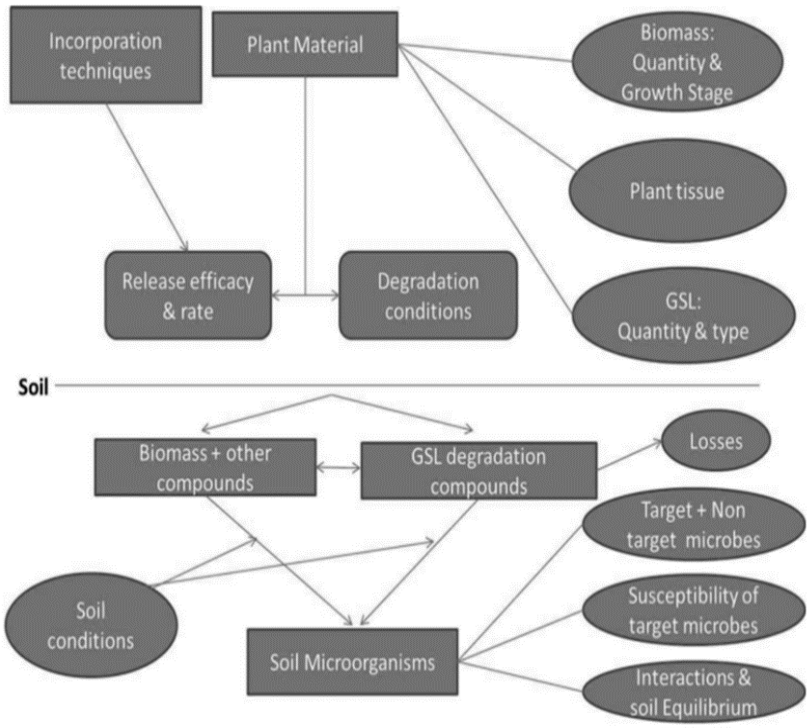


Figure 6. 1 Interlinking of factors affecting the success of soil biofumigation. From (Bellostas *et al.* 2004). GSL – glucosinolates.

Recommendations and future research

The results obtained from the present study can only be used as a preliminary guideline for the fresh produce industry in Australia since there is not enough information on the survival of different *Salmonella* serovars in relation to soil types and environmental factors. In addition, there are no validated remediation strategies that growers could implement to suppress or eliminate the presence of naturally-occurring human pathogens in the soil. Regardless of this, important knowledge has been developed and the future research can be confidently planned (Table 6.1). Based on the abiotic factors that affect the survival of *Salmonella* in the soil (Chapter 4), the field experiment was conducted to evaluate the different treatments (solarisation, biofumigation and solarisation-biofumigation) on the suppression of *Salmonella* in the field together with soil types and climatic factors (Chapter 5). Solarisation was found to be effective in suppressing the pathogen in the soil and clay soil was found to support the survival of *Salmonella* in the soil as it had been observed during the microcosm pot trial. To predict the survival of enteric pathogens in soil, more field studies should be undertaken and validated in different seasons, regions, climates and soil types under Australian conditions. The findings could then be used as a guide for determining safe intervals between the potential contamination of fields with enteric pathogens and the planting or harvest of vegetables. The research presented in this thesis therefore represents an important outcome for the fresh produce industry.

Another point to consider is that the field conditions and crops used in this study may not be replicated in other production systems. A range of combinations of crops types and different soil environments may need to be determined before strict guidelines are developed.

On the basis of these findings, the following recommendations and future research directions can be generated:

- According to the Freshcare Guidelines (2015), the exclusion period between the application of untreated manure and crop harvest is 90 days. In addition, within the exclusion period, fertilisers and soil additives containing manure may be used if subjected to a treatment verified to achieve the benchmark of '*Salmonella* not detected/25 g'. However, in the present study (Chapter 5), *Salmonella* survived more than 100 days in manure-amended clay soil. This suggests that the current guideline should be reconsidered according to soil type.

- The 90 day exclusion period (which also depends on density of pathogen in the manure/compost) between the application of untreated manure and crop harvest remains suitable for farms with sandy or, most probably, sandy loam soils. It may be possible to revise this exclusion period downward for sandy soil types, based on this research but this would need to be verified with further kinetic studies.
- A rapid decline of *Salmonella* in soil can be achieved at relatively high temperatures (>37°C) and is likely to occur during summer months. It should be noted that at high temperatures, a rapid initial decline in *Salmonella* occurs but, based on the data from this study, the rate of inactivation thereafter it is not temperature-dependent and that *Salmonella* can still persist in soil in low numbers for an extended period of time.
- Moisture fluctuation can lead to a faster decline in *Salmonella* survival in soil, with or without manure amendment, compared with constant moisture conditions. Therefore, withholding irrigation before critical period for irrigation that will not affect the crop (for instance, before head development in lettuce crop), may hasten die-off of *Salmonella*.
- Soil solarisation with black plastic may be used to remediate *Salmonella*-contaminated soil providing temperatures reach 37°C for extended periods of time. The results from this study indicate that further investigation of temperature and time relationships for soil solarisation treatment is warranted.
- Cover crop coverage, stages of incorporation and degree of maceration of the cover crops needs to be assessed so that enough biomass production and biofumigation potential for the suppression of *Salmonella* in the soil is achieved. In addition, management practices and environmental conditions that maximise total glucosinolate production and isothiocyanate efficiency in amended soil need to be investigated.
- Biocidal compounds produced by biofumigant cover crops need to be accurately identified and quantified, and the optimum levels to achieve an antimicrobial effect need to be determined.
- An evaluation of bacterial pathogens and their potential to attach and internalise plants through their roots needs to be further investigated, given the pathogen remained in the soil throughout the study.

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8. Appendices

Appendix 1 Preparation of media

1.1 Preparation of nutrient agar

Nutrient agar (NA) medium was prepared by suspending 9.2 g dehydrated NA (Difco™, BD, Sparks, USA) in 400 mL distilled water in a bottle and boiled to completely dissolve. The medium was sterilised by autoclaving at 121°C for 20 min. The medium was cooled to approximately 50°C and poured into sterile Petri dishes which were stored at 5°C until required.

1.2 Preparation of trypticase soy agar

Trypticase soy agar (TSA) medium was prepared by suspending 16 g dehydrated TSA (Difco™, BD, Sparks, USA) in 400 mL distilled water in a bottle and boiled to completely dissolve. The medium was sterilised by autoclaving at 121°C for 20 min. The medium was cooled to approximately 50°C and poured into sterile Petri dishes which were stored at 5°C until required.

1.3 Preparation of tryptic soy broth

Tryptic soy broth (TSB) medium was prepared by suspending 30 g dehydrated TSB (BBL™, BD, Sparks, USA) in 400 mL distilled water. The medium was heated to completely dissolve and sterilised by autoclaving at 121°C for 20 min. The broth was stored at 5°C until required.

1.4 Preparation of xylose lysine deoxycholate agar

Xylose lysine deoxycholate *(*XLD) agar was prepared by suspending 22 g dehydrated XLD agar (Difco, BD, Sparks, USA) in 400 mL distilled water. The medium was heated to boiling (using a 240 W microwave, medium power 2 min; high power 2 min; medium power 2 min) with frequent agitation to completely dissolve all solids without overheating. The medium was cooled to approximately 50°C and poured into sterile Petri dishes which were stored at 5°C until required.

1.5 Preparation of xylose-lysine-tergitol 4 agar and agar supplement

Xylose-lysine-tergitol 4 (XLT4) with agar supplement was used for presumptive isolation and differentiation of *Salmonella* (Miller *et al.* 1991). XLT4 agar medium was prepared by suspending 23.6 g dehydrated XLT4 (Difco, BD, Sparks, USA) agar and 1.84 mL agar supplement in 400 mL distilled water. The medium was heated to boiling as described in 3.3.5. The medium was cooled to approximately 50°C and poured into sterile Petri dishes which were stored at 5°C until required. Typical *Salmonella* colonies (H_2S-positive) appear black or black-centered with a yellow periphery after 18–24 h of incubation. Upon continued incubation, the colonies become entirely black or pink to red with black centres. Colonies of H_2S-negative strains appear pinkish yellow.

1.6 Preparation of phosphate buffer stock solution

Phosphate buffer, pH 7.2, was used for the preparation of dilution blanks and washing lawn cultures. A stock solution was prepared by dissolving 34 g of dehydrated phosphate buffer (BBL™, BD, Sparks, USA) in 1 L distilled water. A working solution was prepared by diluting 1.25 mL stock solution to 1 L with distilled water in a volumetric flask. The working solution was sterilised by autoclaving at 121°C for 20 min and was stored at 5–25°C for use.

1.7 Preparation of mBroth

mBroth (Bacto, BD, Sparks, USA) was prepared by suspending 14.5 g dehydrated medium in 400 mL distilled water. The medium was heated on a hot plate with a magnetic stirrer to dissolve completely prior to autoclaving at 121°C for 20 min. The mBroth solution was stored at 5°C until required.

1.8 Preparation of Demi-Fraser broth and Fraser supplement

Demi-Fraser broth was prepared by suspending 22 g of the dehydrated medium (Accumedia, USA) in 400 mL distilled water. The medium was heated on a hot plate with a magnetic stirrer to completely dissolve the medium prior to sterilisation by autoclaving at 121°C for 20 min. Demi-Fraser broth was stored at 5°C and before use, the broth was brought to room temperature and 4 mL of Demi-Fraser supplement was added to 400 mL broth. The solution was mixed by hand and used immediately.

1.9 Preparation of *Listeria* chromogenic agar

Listeria chromoagenic agar (Conda, Spain) was prepared by suspending 35.275 g dehydrated media in 500 mL of distilled water. The medium was mixed well and dissolved by heating with frequent agitation. The medium was sterilised by autoclaving at 121°C for 20 min. The medium was cooled to approximately 45–50ºC and 5 mL of *Listeria* Lipase C supplement was aseptically added. *Listeria* chromogenic selective supplement, which was previously reconstituted in 5 mL sterile water/acetone (1:1), was also added to the medium. The medium was mixed well and poured into sterile Petri dishes and stored at 5°C until required.

1.10 CHROMagar *Salmonella*

CHROMagar *Salmonella*, purchased in pre-prepared plates (Micromedia, Edwards, Australia), is a chromogenic selective and differential medium for the presumptive identification of *Salmonella* species. *Salmonella* species produce rose-to-purple colonies that are easily differentiated from other bacteria that appear blue or unstained and may resemble *Salmonella* on traditional media.

Appendix 2 Method of statistical analysis for the microcosm pot trial

Fixed effects tests

Source	Nparm	DFNum	DFDen	F Ratio	Prob>F
Week	6	6	1867.0	60.588983	<0.0001*
Temp	2	2	1867.0	50.539814	<0.0001*
Soil	1	1	1867.0	1087.6737	<0.0001*
Manure	1	1	1867.0	492.09063	<0.0001*
Serovar	3	3	1867.0	28.781184	<0.0001*
Moisture	1	1	1867.0	333.26252	<0.0001*
Week*Temp	12	12	1867.0	6.2183167	<0.0001*
Week*Soil	6	6	1867.0	12.922282	<0.0001*
Week*Manure	6	6	1867.0	4.7960356	<0.0001*
Week*Serovar	18	18	1867.0	1.779691	0.0226*
Week*Moisture	6	6	1867.0	10.838326	<0.0001*
Temp*Soil	2	2	1867.0	20.197795	<0.0001*
Temp*Manure	2	2	1867.0	63.478819	<0.0001*
Temp*Serovar	6	6	1867.0	11.555592	<0.0001*
Temp*Moisture	2	2	1867.0	9.7095725	<0.0001*
Soil*Manure	1	1	1867.0	23.279052	<0.0001*
Soil*Serovar	3	3	1867.0	0.8924306	0.4443
Soil*Moisture	1	1	1867.0	4.807304	0.0285*
Manure*Serovar	3	3	1867.0	1.368476	0.2507
Manure*Moisture	1	1	1867.0	16.205979	<0.0001*
Serovar*Moisture	3	3	1867.0	0.4938356	0.6866

Example of multiple comparisons for temperature and soil

Temp	Soil	Estimate	Std Error	DF	t Ratio	Prob>|t|
5	clay	3.8900020	0.05851399	1867	66.48	<0.0001*
5	sand	1.9376410	0.05831178	1867	33.23	<0.0001*
21	clay	3.4826787	0.05910447	1867	58.92	<0.0001*
21	sand	1.8879526	0.05792588	1867	32.59	<0.0001*
37	clay	2.9276539	0.05984299	1867	48.92	<0.0001*
37	sand	1.7239514	0.05895925	1867	29.24	<0.0001*

Tukeys HSD All Pairwise Comparisons

Quantile = 2.8527, Adjusted DF = 1867.0, Adjustment = Tukey-Kramer

Temp	Soil	Temp	Soil	Difference	Std Error	t Ratio	Prob>|t|
5	clay	5	sand	1.95236	0.0826142	23.63	<0.0001*
5	clay	21	clay	0.40732	0.0831126	4.90	<0.0001*
5	clay	21	sand	2.00205	0.0823325	24.32	<0.0001*
5	clay	37	clay	0.96235	0.0836788	11.50	<0.0001*
5	clay	37	sand	2.16605	0.0830729	26.07	<0.0001*
5	sand	21	clay	-1.54504	0.0830211	-18.61	<0.0001*
5	sand	21	sand	0.04969	0.0821937	0.60	0.9907
5	sand	37	clay	-0.99001	0.0835557	-11.85	<0.0001*
5	sand	37	sand	0.21369	0.0829189	2.58	0.1034
21	clay	21	sand	1.59473	0.0827609	19.27	<0.0001*
21	clay	37	clay	0.55502	0.0840915	6.60	<0.0001*
21	clay	37	sand	1.75873	0.0835055	21.06	<0.0001*
21	sand	37	clay	-1.03970	0.0832787	-12.48	<0.0001*
21	sand	37	sand	0.16400	0.0826557	1.98	0.3518
37	clay	37	sand	1.20370	0.0840146	14.33	<0.0001*

Example of multiple comparisons for week number

Week No	Estimate	Std Error	DF	t Ratio	Prob>|t|
0	4.4777191	0.13056852	23.417	34.29	<0.0001*
1	5.4134367	0.13056852	23.417	41.46	<0.0001*
2	4.5287318	0.13056852	23.417	34.68	<0.0001*
3	4.1005030	0.13056852	23.417	31.40	<0.0001*
4	3.4549246	0.13056852	23.417	26.46	<0.0001*
5	3.2485914	0.13056852	23.417	24.88	<0.0001*

Appendix 3 Method of statistical analysis used for the field experiment

Example of multiple comparisons for split-plot (solarisation and control)

Split-plot	Estimate	Std Error	DF	t Ratio	Prob>|t|
fallow-open	1.6819247	0.21515254	11.346	7.82	<0.0001*
fallow-solarisation	0.5568268	0.21515254	11.346	2.59	0.0252*
mustard-open	1.6435892	0.21515254	11.346	7.64	<0.0001*
mustard-solarisation	1.2582584	0.21515254	11.346	5.85	0.0001*
radish-open	1.4344389	0.25788405	4.6703	5.56	0.0051*
radish-solarisation	1.3901180	0.25788405	4.6703	5.39	0.0057*
sorghum-open	1.8656369	0.22597509	12.288	8.26	<0.0001*
sorghum-solarisation	0.5397265	0.22597509	12.288	2.39	0.0342*

Appendix 4 Proportion of *Salmonella* cells recovered after direct plating and enrichment

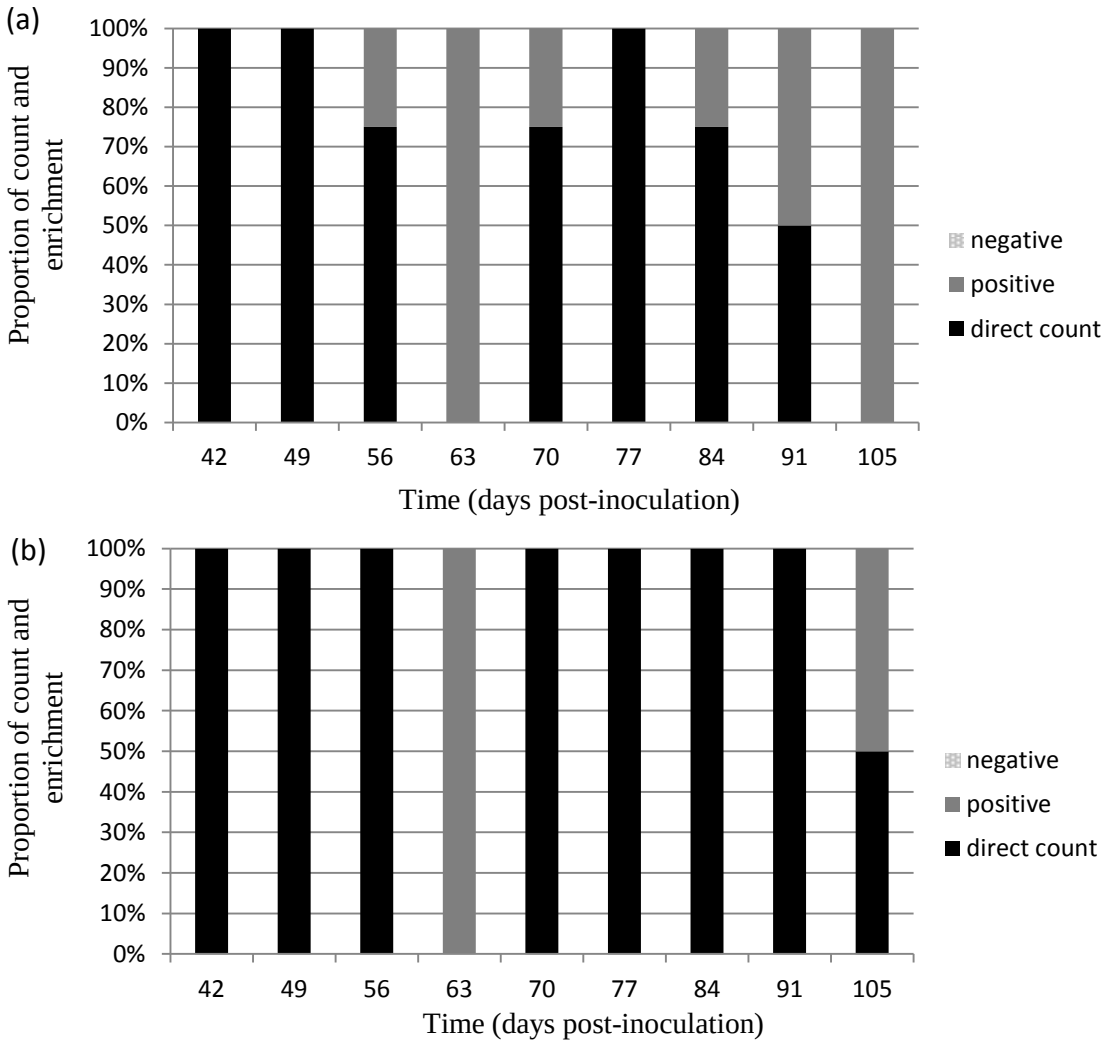

Figure A4.1 The proportion of *Salmonella* cells recovered using direct plating and enrichment (positive or negative) from (a) mustard-solarisation and (b) mustard-open treatments at Pye Farm (clay soil).

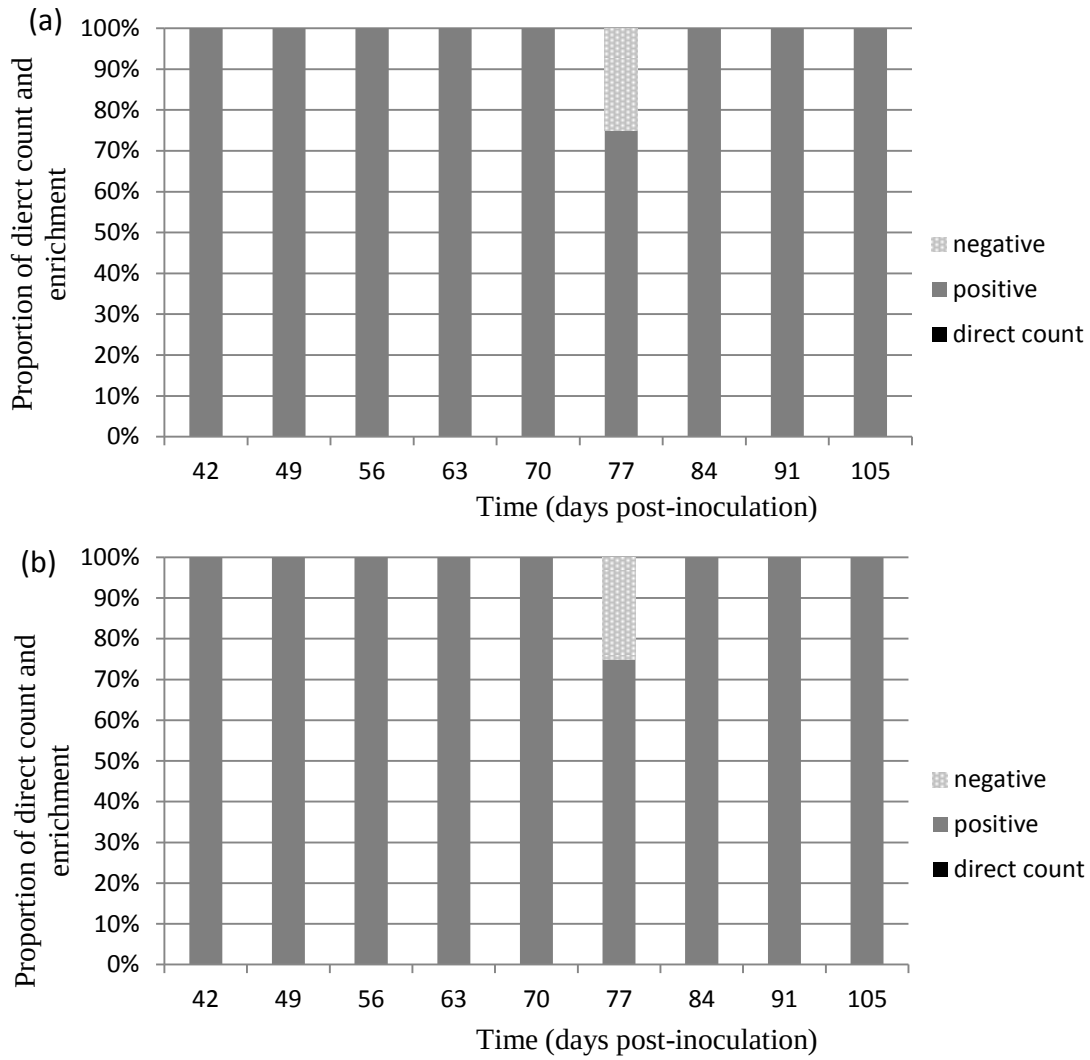

Figure A4.2 The proportion of *Salmonella* cells recovered using direct plating and enrichment (positive or negative) from (a) mustard-solarisation and (b) mustard-open treatments at Karalee Farm (sandy soil).

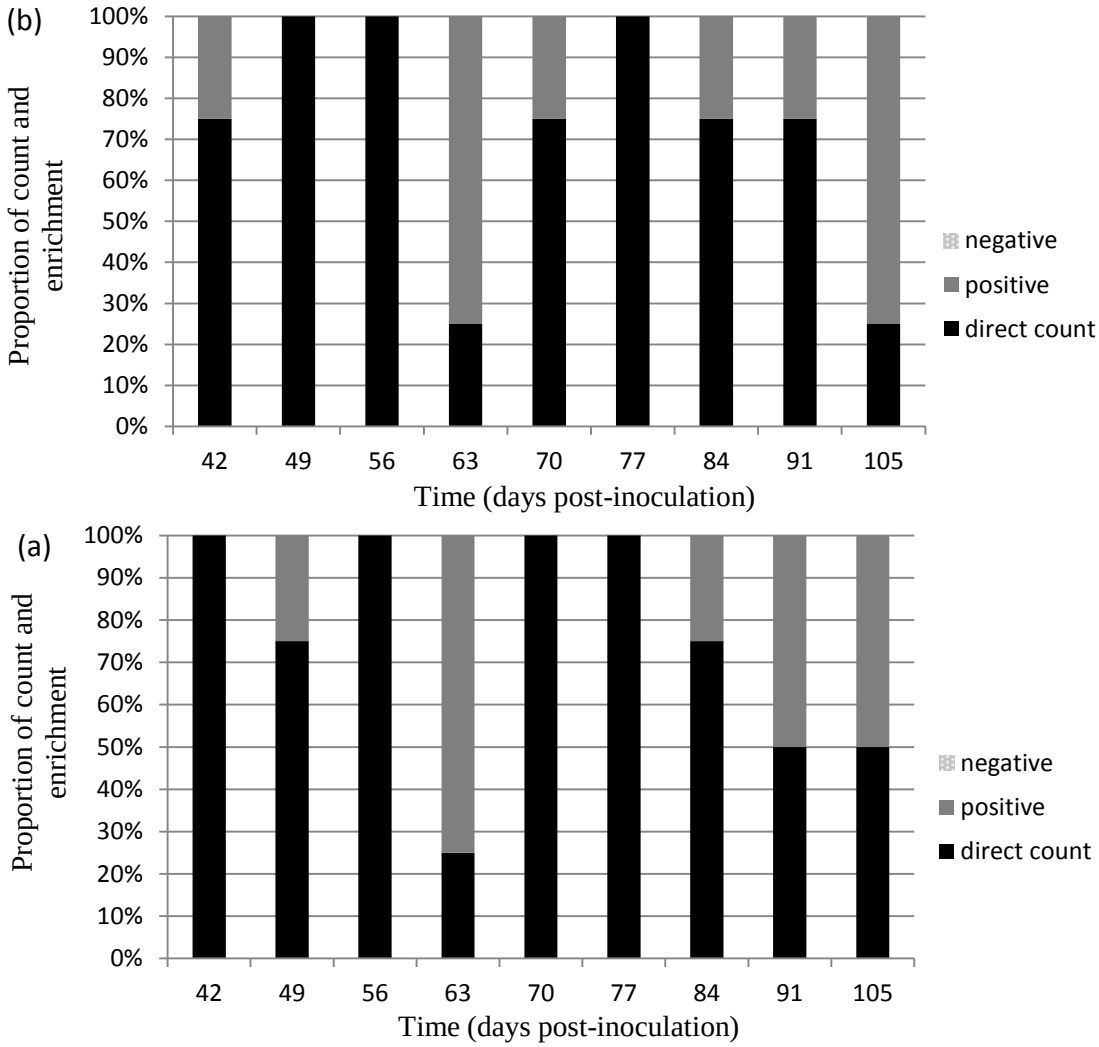

Figure A4.3 The proportion of *Salmonella* cells recovered using direct plating and enrichment (positive or negative) from (a) radish-solarisation and (b) radish-open treatments at Pye Farm (clay soil).

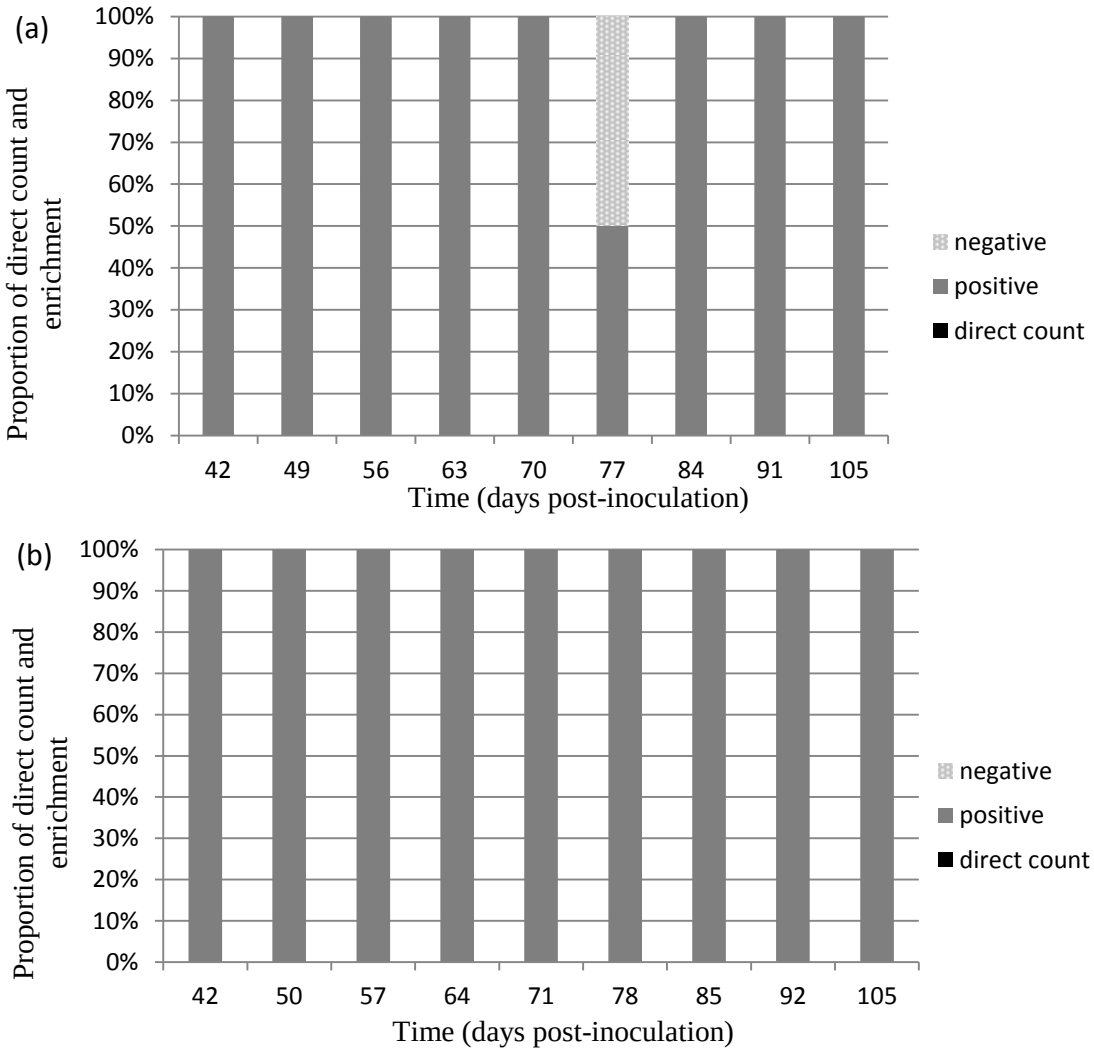

Figure A4.4 The proportion of *Salmonella* cells recovered using direct plating and enrichment (positive or negative) from (a) radish-solarisation and (b) radish-open treatments at Karalee Farm (sandy soil).